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The Distribution of Glycogen in the Liver of Rabbits

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(*Received* 28 *March* 1949)

Attention has recently been called by Gomori & Goldner (1947) to a strikingly uneven distribution of glycogen in the liver of rabbits. According to these authors, variations exceeding several 100 % may be observed in simultaneous biopsies taken from the same or from different liver lobes.

In the present investigation, a survey of the distribution of liver glycogen has been carried out on rabbits submitted to a variety of experimental conditions. It has not been possible to confirm Gomori & Goldner's (1947) observations. Only relatively small intralobar and interlobar differences were observed, not exceeding those described in other animal species.

Only the distribution of glycogen will be considered here. The effects of the various experimental conditions on the glycogen content will be described elsewhere.

METHODS

Ten rabbits were chosen for the survey by sampling a very heterogeneous population, including fed and fasting (24 hr.) animals, and fasted animals injected with anterior pituitary extract, or with glucose + insulin or with both (two animals in each group). Their liver glycogen content varied between 0·32 and 8·55 %.

The animals were killed by intravenous injection of 500 mg. sodium-Evipan. Samples weighing approximately 100 mg. were taken immediately after death and immersed in tared centrifuge tubes containing 2 ml. of 30 % (w/v) KOH. They were analysed according to Good, Kramer & Somogyi (1933), the final glucose determination being made by the method of Nelson (1944).

The sampling was made on the three main lobes of the liver: 1 = right lobe (+ gall-bladder), 2 = middle lobe, 3 = left lobe. Three samples were taken from distant sites in each lobe. Three contiguous samples were further taken from one of the lobes for estimation of the error of determination.

Interlobar and intralobar variations were determined statistically by making a variance analysis of the results (Mather, 1946). In order to homogenize the variances from animals which were not immediately comparable, the analysis was made on the logarithms of the results, using natural logarithms. An estimation of the relative variations of the glycogen content throughout the liver was thereby obtained. It may be pointed out that the absolute differences between comparable results were found to increase with increasing glycogen contents, whereas the relative differences remained approximately constant.

RESULTS

The results of the variance analysis are summarized in Table 1.

Table 1. *Variance analysis of distribution of liver glycogen*

Origin of variance	Degrees of freedom	Sum of squares	Mean square	P	Relative S.D.* (%)
Rabbits + treatment	9	36·9722	—	—	—
Lobes	20	0·2558	0·0128	<0·001	8·6
Position in lobe	60	0·2332	0·0039	<0·001	6·4
Analytical procedure†	60	0·0692	0·00115	—	3·45

* Gives the equivalent of the logarithmic standard deviation from the mean, in percentage of the mean.

† Since only one of the nine determinations made on each animal was done in triplicate, the estimation of this factor could not be included in the general variance analysis, but was effected separately.

For the purpose of illustration, the results of the two experiments in which the largest discrepancies were observed have been recorded fully in Table 2.

Table 2. *Distribution of liver glycogen*

			Glycogen content (g./100 g. wet wt.)				
Rabbit no.	Treatment	Lobe	Site A		Site B	Site C	Mean of lobe	Mean of liver
8	Fasting (24 hr.)	1	1·34		1·00	1·21	1·18	1·24
		2	1·40 1·35 1·20	1·32	1·29	1·22	1·28	
		3	1·24		1·13	1·39	1·25	
9	Fed	1	5·70		5·10	4·76	5·19	5·24
		2	5·10 5·51 5·60	5·40	6·35	5·71	5·82	
		3	4·71		4·52	4·93	4·72	

(*a*) *Error of determination.* This includes possible local differences between contiguous pieces of liver tissue. As is shown in Table 1, the standard deviation

from the mean of three results obtained on contiguous samples amounts to 3·45 % of the mean value.

(*b*) *Intralobar distribution.* When three pieces of liver are taken from distant sites in the same lobe, the variations are significantly larger than those arising only from determination errors (relative S.D. = 6·4 %, $P < 0·001$). These variations are, however, much smaller than those described by Gomori & Goldner (1947). It may be calculated that the probability of finding a local glycogen content more than 16 % higher or lower than the average content of the lobe is smaller than 0·01. Out of the sixty determinations of this kind which have been made, none shows a deviation of that magnitude and only three deviate more than 10 % from the mean lobar value, 10, 14·4 and 15·5 % respectively.

(*c*) *Interlobar distribution.* Interlobar variations are significantly higher than the intralobar ones (relative S.D. = 8·6 %, $P < 0·001$), but here again the differences are not very important quantitatively. It may be computed from the data presented that a single glycogen determination, performed on 100 mg. of hepatic tissue, would, under the conditions of our experiments, be representative of the average glycogen content of the whole liver with a standard error of 8·6 %. The probability that the result obtained would exceed the average content by more than 25 % is smaller than 0·01.

Closer analysis of the individual results shows that the heterogeneity of the lobar distribution is almost entirely due to the middle lobe (lobe 2), whose glycogen content is systematically higher than those of the two others, which are usually in close agreement (average increase for lobe 2: 5·9 %; limits for $P = 0·01$: 2·4 and 9·5 %).

(*d*) *Individual differences and differences due to treatment.* It should be mentioned here that the results were first analysed separately for each individual rabbit and then by groups of two similarly treated animals. Comparison of the results of the individual and group analyses showed that the relative distribution of glycogen in the liver was not significantly affected either by individual differences nor by differences due to the treatment. A bulk analysis of the homogenized variances of all the animals was thereby justified. Only the results of this analysis have been given in Table 1.

DISCUSSION

In view of the data presented here, there seems to be no reason to exclude the rabbit from studies involving determinations of liver glycogen. An analysis performed on three pieces taken at random in the three lobes of the liver should furnish a very satisfactory estimation of the average glycogen content of that organ.

It is difficult to reconcile these results with those of Gomori & Goldner (1947). However, the conclusions of these authors are partly based on histological evidence, and one may wonder to what extent microscopic heterogeneity is liable to affect the average glycogen content of a macroscopic sample of tissue.

SUMMARY

1. The distribution of glycogen in the liver of rabbits subjected to a variety of experimental conditions has been studied chemically and the results analysed statistically.

2. The distribution in the same lobe and in different lobes has been found to be significantly, though moderately, heterogeneous.

3. The relative S.D. of a single determination with respect to the average glycogen content of the whole liver was found to be 8·6 % under the conditions of our experiments.

This work has been supported by a grant from the Lilly Research Laboratories.

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Prosthetic Groups of the Cytochromes Present in *Corynebacterium diphtheriae* with Especial Reference to Cytochrome *a*

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(*Received* 28 *December* 1948)

As a preliminary to investigations on porphyrin synthesis by *Corynebacterium diphtheriae* with various isotopically labelled compounds, we deemed it essential to study the prosthetic group of the cytochromes of this organism. The present paper reports the results so far obtained.

With *C. diphtheriae*, controlled improvement of toxin production has led to a corresponding increase in the production of free porphyrin; accurate adjustment of iron concentration is essential for the production of optimum amounts of toxin, even when other factors such as pH, nutrients, etc. are optimal. Pappenheimer (1947*a*, *b*) has shown that there is a parallel rise and fall of toxin and porphyrin excreted into the medium during cultivation under different conditions. The porphyrin was identified tentatively by Campbell-Smith (1930) and Coulter & Stone (1931) and was characterized as coproporphyrin III by Gray & Holt (1947). A functional relationship between toxin and pigment was postulated by Pappenheimer (1947*a*) after he had obtained evidence to indicate that for every 4 atoms of iron added above the amount giving an optimum yield of toxin, there failed to appear in the culture fluid 4 mol. of porphyrin and 1 mol. of toxin. At such higher iron levels he found that cells contained greater amounts of a haemochromogen-like substance. *C. diphtheriae* contains large amounts of cytochrome *b* and smaller amounts of *c* and *a* (Fujita & Kodama, 1934). In the present work the prosthetic group of cytochrome *b* has been identified as protohaem no. 9 and the prosthetic group of cytochrome *a*, although it has not been identified with any structure of established constitution, has been investigated in some detail.

EXPERIMENTAL

Measurement of absorption spectra. The wave lengths of absorption maxima were measured with a Beck-Hartridge reversion spectrometer. In the case of intact cell suspensions, additional measurements were made by means of a Zeiss microscope eyepiece spectrometer.

For the measurement of optical densities, a Beckman photoelectric spectrophotometer was used. When dealing with solutions of pyridine haemochromogens, it was necessary to take the following precautions. The 1 cm. glass cell was filled completely with solution and the lid fitted without the introduction of air bubbles. The solid $Na_2S_2O_4$ used as reducing agent was dissolved by rotating the cell. On account of the variability of different batches of $Na_2S_2O_4$ the minimum amount required to effect complete reduction was found by trial. In order to prevent turbidity appreciable excess of $Na_2S_2O_4$ was avoided. Measurements at wave lengths shorter than 400 mμ. were not accurate as $Na_2S_2O_4$ shows significant absorption in this region.

Determination of iron in haems. The *o*-phenanthroline method of Drabkin (1941) was found to be satisfactory, but it was necessary to use twice the amount of ascorbic acid prescribed by him. With that modification and measuring optical densities at 510 mμ. (Beckman spectrophotometer) a straight-line relationship was found to obtain for solutions containing 0–100 μg. Fe/25 ml.

Cultivation of organisms. Two strains of *C. diphtheriae* were used: (*a*) Park Williams no. 8, substrain Toronto; (*b*) a variant of (*a*) supplied by Mr L. B. Holt of the Wright-Fleming Institute of Microbiology, London; the organisms were grown on the casein hydrolysate medium described by Holt (1948), the Fe concentration being either that found necessary for optimum toxin production (0·14μg./ml.) or 10 times that amount. In one instance a large batch (180 l.) was grown on beef papain digest medium.

In all cases the cells were harvested by centrifuging and washed 3 times with saline before further treatment.

RESULTS

Spectroscopic investigation of intact C. diphtheriae *cells grown at* Fe *level optimal for toxin production*

Aerated, thick suspensions of washed cells in phosphate buffer (pH 7·6) showed no definite absorption bands. Reduction with a small amount of $Na_2S_2O_4$ revealed bands at about *600*, 565 and *532* mμ., the first and last bands being of low intensity (figures given in italics indicate relatively weak absorption). The addition of pyridine, to give 25% (v/v) final concentration, brought about a shift of band positions to *590–580*, 557 and *527* mμ.

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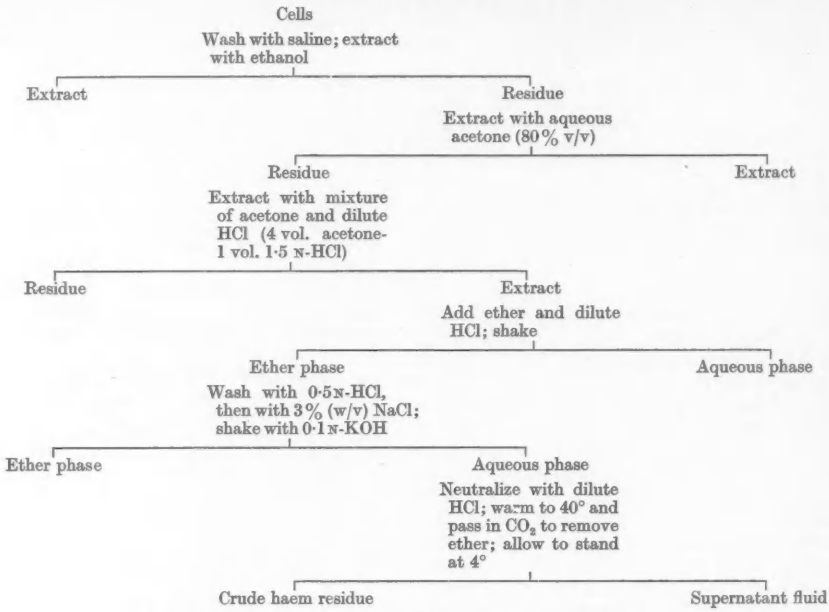

Cells
Wash with saline; extract with ethanol
Extract
Residue
Extract with aqueous acetone (80% v/v)
Residue
Extract
Extract with mixture of acetone and dilute HCl (4 vol. acetone-1 vol. 1·5 N-HCl)
Residue
Extract
Add ether and dilute HCl; shake
Ether phase
Aqueous phase
Wash with 0·5N-HCl, then with 3% (w/v) NaCl; shake with 0·1 N-KOH
Ether phase
Aqueous phase
Neutralize with dilute HCl; warm to 40° and pass in CO_2 to remove ether; allow to stand at 4°
Crude haem residue
Supernatant fluid

Fig. 1. Separation of intracellular haems from *C. diphtheriae*.

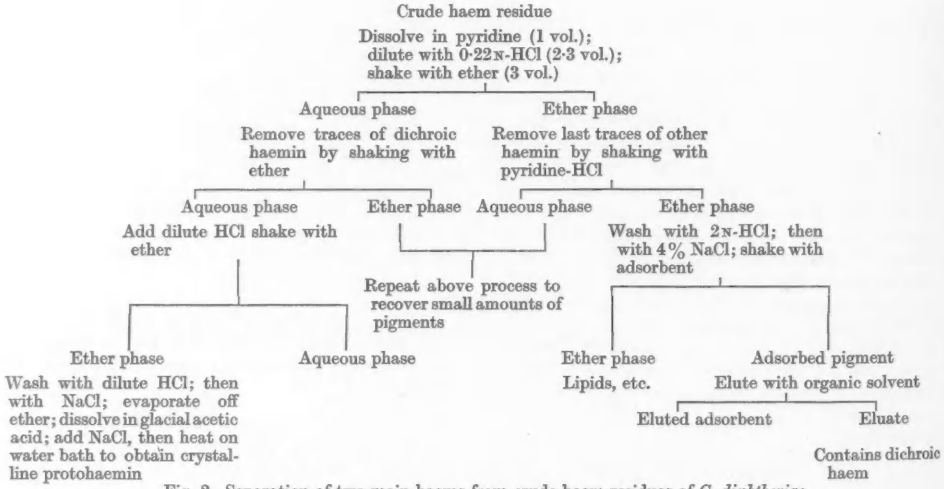
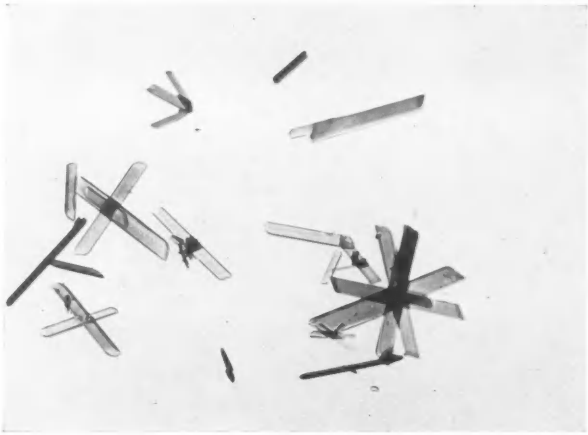

Crude haem residue
Dissolve in pyridine (1 vol.); dilute with 0·22N-HCl (2·3 vol.); shake with ether (3 vol.)
Aqueous phase
Ether phase
Remove traces of dichroic haemin by shaking with ether
Remove last traces of other haemin by shaking with pyridine-HCl
Aqueous phase
Ether phase
Aqueous phase
Ether phase
Add dilute HCl shake with ether
Wash with 2N-HCl; then with 4% NaCl; shake with adsorbent
Repeat above process to recover small amounts of pigments
Ether phase
Aqueous phase
Ether phase
Adsorbed pigment
Wash with dilute HCl; then with NaCl; evaporate off ether; dissolve in glacial acetic acid; add NaCl, then heat on water bath to obtain crystalline protohaemin
Lipids, etc.
Elute with organic solvent
Eluted adsorbent
Eluate
Contains dichroic haem

Fig. 2. Separation of two main haems from crude haem residues of *C. diphtheriae*.

Cells grown at higher iron levels inhibitory to toxin production

In respect of spectral pattern these cells were similar to those at a lower Fe level but the band intensities were at least five times as great. This enabled us to measure accurately the band positions which were (in mμ.): reduced cell suspension, 600; 568...554 (maximum at 563); reduced cell suspension, aerated (faint shading), 560...550; reduced



(*a*) Crystalline protohaemin from *C. diphtheriae* cells. × 100.

(*b*) Pyridine haemochromogen crystals from protohaemin of *C. diphtheriae* cells. × 100.

(*c*) Pyridine protohaemochromogen crystals. × 100.

cell suspension and addition of pyridine, 599...582 (maximum at 590); 564...549 (maximum at 556); 522 (diffuse).

This result indicated the presence of two principal intracellular haem components and we concluded that there was an increase in cytochromes *b* and *a* complex in cells grown at higher iron concentrations (cf. Pappenheimer, 1947*a*, *b*; Pappenheimer & Hendee, 1947).

Extraction and separation of intracellular haems from Corynebacterium diphtheriae

As attempts to extract the intact haemproteins were not successful, it was decided to extract the haem prosthetic groups by means of acid acetone. The flow sheet (Fig. 1) illustrates the process used.

Tests on the extracts confirmed the presence of haems exhibiting haemochromogen bands at 587, 556 and 522 mμ. The process outlined in Fig. 2 gave clean separation if attention was paid to the number of washings given to each phase. The removal of final traces of unwanted pigments was checked, after each washing, by haemochromogen tests on small samples.

The dichroic haem was either adsorbed on charcoal and eluted with pyridine, or it was adsorbed on alumina, in which case elution with warm glacial acetic acid was found to be more effective.

Table 1. *The absorption spectra of protohaemin from cells of* Corynebacterium diphtheriae *compared with authentic protohaemin no.* 9

(The two crystalline haemin samples were dissolved in acetone solution (0·3N with respect to HCl), transferred into ether, washed with 2N-HCl and then with water before transfer into 0·1N-KOH. The mixtures were diluted finally with 0·5 vol. of pyridine.)

Solvent	Protohaemin: *C. diphtheriae* (mμ.)	Protohaemin: Authentic no. 9 (mμ.)
Acetone-HCl	642	642
	*589**	*590*
	546	547
	505	505
Ether (HCl washed)	638	638
	586	*586*
	544	544
	503	503
KOH (0·1N)	615	615
	575	576
KOH (0·1N) + $Na_2S_2O_4$	580	580
	543	544
KOH (0·1N) + $Na_2S_2O_4$ + pyridine	556	556
	524	523
	487	*486*

* Figures in italics indicate relatively weak absorption.

Characteristics of the haem passing into the aqueous phase

The pigment from the aqueous phase, obtained in the form of Teichmann crystals (Pl. 2*a*), gave with Takajama reagent crystals (Pl. 2*b*) identical with those of pyridine-protohaemochromogen (Pl. 2*c*). Table 1 records a comparison between this haemin and authentic crystalline protohaemin no. 9.

The quantitative spectral absorption curve (based on iron content, Fig. 3) further illustrates the identity of the haemin with protohaemin. The iron content of the crystalline product was 96% of that of protohaemin prepared from ox blood and after recrystallization this value increased to 97%.

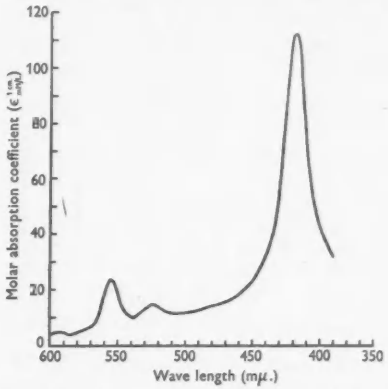


Fig. 3. Absorption spectrum of pyridine protohaemochromogen from *C. diphtheriae*.

Removal of iron. The $SnCl_2$-HCl method (Hamsik, 1931) was suitable for acetone solutions of the pigment, but for general purposes the ferrous acetate method of Warburg & Negelein (1932) was preferred. In Table 2 a comparison is made between

Table 2. *The absorption spectra of protoporphyrin derived from protohaemin of* Corynebacterium diphtheriae *cells compared with pure protoporphyrin no.* 9

Solvent	Protoporphyrin: *C. diphtheriae* (mμ.)	Protoporphyrin: Pure no. 9 (mμ.)
Ether	632	632
	580	578
	537	537
	505	505
HCl (5 g. HCl/100 ml.)	601	600
	580	579
	556	555
$CHCl_3$ (from acidic solution)	605	604
	*585**	*584*
	559	559

* Figures in italics indicate relatively weak absorption.

the porphyrin obtained and authentic protoporphyrin no. 9.

Conversion of the haemin to mesoporphyrin. The hydriodic acid method of Fischer & Kögl (1924) was used. The mesoporphyrin was esterified with methanol saturated with hydrogen chloride, the ester purified in the usual way and crystallized four times from chloroform-methanol. A sample of protohaemin no. 9 was treated similarly. The mesoporphyrin methyl ester crystals obtained were identical in form and gave similar melting points (207° uncorr.). Spectrometric comparisons are given for the free porphyrins and methyl esters in Table 3.

Table 3. *The absorption spectra of mesoporphyrin derived from protohaemin of* Corynebacterium diphtheriae *cells compared with authentic mesoporphyrin no. 9*

	Mesoporphyrin	
Solvent	*C. diphtheriae* (mμ.)	Authentic no. 9 (mμ.)
Ether	623	623
	598	599
	578	580
	568	568
	527	527
	497	496
HCl (25 g. HCl/100 ml.)	594	593
	*573**	*574*
	550	550
$CHCl_3$† (from acidic solution)	595	595
	575	575
	551	552
	—	*527*
	496	490
$CHCl_3$† (washed with NH_3 soln., 12% diln. of conc.)	620	620
	595	*595*
	576	576
	567	567
	532	532
	500	500

* Figures in italics indicate relatively weak absorption.
† After conversion into dimethyl ester.

Characteristics of the haem from the ether phase

Solutions of this pigment were dichroic, concentrated solutions being red, while dilute solutions were yellow green. The pyridine-haemochromogen test on this haem showed a strong band at 587, a weak band at 553 and a very faint band at 525 mμ. (see Fig. 4). This indicated that the pigment contained more than one component. The 553 and 525 mμ. bands were observed to increase in intensity relative to the 587 mμ. band when solutions of this fraction were left standing. The absorption curve was therefore assumed to be the resultant of a main component with absorption maximum at 587 mμ., and a product derived from it with bands at 553, 525 and about 418 mμ. The addition of cysteine hydrochloride to a solution of the pyridine-haemochromogen mixture caused the slow disappearance of the 587 mμ. band and a simultaneous appearance of extra bands close to the 553 and 525 mμ. bands.

Removal of iron. The method of Warburg & Negelein (1932) was used to remove the iron from this fraction and an inspection with the reversion spectrometer clearly revealed the presence of two sets of porphyrin bands. These were (pyridine solution): I (*a*) 645, (*b*) 627; II, 593...581; III (*a*) 559, (*b*) 541; IV, 505 mμ. Order of intensity: III (*a*), II, IV, I (*a*), I (*b*), III (*b*). A partial separation of the two components was achieved by extraction from ether solutions by means of hydrochloric acid solutions of different concentration. Neither porphyrin was readily extractable by solvents less acidic than 3N-HCl.

Comparison of the a component from Corynebacterium diphtheriae *cells with that from heart muscle*

At this stage it was decided to reconsider the value of further investigations on the pigments in this ether fraction. Although partial separation of the two porphyrins was possible by differential acid extraction, the extra manipulations involved and the difficulties experienced in eliminating reactive substances carried over from the original extracts led us to attack the problem in a different manner. The justification for this decision will be discussed below, but for the sake of continuity a brief comment is needed. The pigment responsible for the 587 and 430 mμ. pyridine haemochromogen bands was assumed to be derived from the cytochrome *a* complex. It was therefore considered worth while to investigate another tissue (ox-heart muscle) known to be rich in this component.

Modifications of extraction and separation processes used in isolating the dichroic haem a from heart muscle. Ox hearts were minced after freeing them from fatty and ligamentous tissue and then given either a preliminary dehydration with ethanol or extracted directly with acetone. Either anhydrous or 80% (v/v) aqueous acetone was equally satisfactory. A mechanical press was used to remove fluid, and extractions were continued until yellow pigments were no longer apparent (normally three extractions). The intracellular haems were extracted as before, care being taken to compensate for acid bound to tissue material during the first extraction. We have used successfully for first extractions mixtures with final concentrations 0·3N with respect to HCl and ranging from 80 to 98% aqueous acetone. With subsequent extractions less HCl was required to maintain acidic conditions in the extraction fluid. The greater part of the haem pigments was extracted rapidly, prolonged treatment being necessary to recover further significant amounts. Extractions were made at 4° to minimize the risks of decomposition.

From extracts containing little water and HCl, the pigments were precipitated by addition of saturated sodium acetate solution. The crude haem residue obtained by washing the precipitate with acetone and then dilute acetic

acid was now ready for solution in pyridine and isolation. This technique was most useful in removing lipid matter. If the extracts would not yield precipitated haem, by such direct treatment, the pigments were transferred into ether by the process outlined in Fig. 2, transfer into dilute alkali being found unnecessary.

The aqueous pyridine phase contained at least one other haem compound in addition to protohaem, as haemochromogen tests showed two additional bands at wave lengths slightly lower than the α and β bands of protohaemochromogen. The dichroic haem was adsorbed by passing the washed ether phase through a column of alumina after which the alumina was washed well with fresh ether. By stirring the dry residue with warm glacial acetic acid (50–60°) the haem was eluted to give a dark brown solution. In this condition the pigment remained stable even after long standing at room temperature.

Characteristics of haem a *isolated from ox-heart muscle.* The results of spectroscopic investigations of this haem are summarized in Table 4.

Pyridine haemochromogen a. The quantitative absorption curve illustrated in Fig. 4 was obtained by dissolving a sample of the dichroic haem, containing 34·9 μg. Fe in 6·25 ml. pyridine (A.R.) and 12·5 ml. 0·1 N-NaOH and adding $Na_2S_2O_4$. This solution was diluted to 25 ml. with distilled water and measurements were made with a Beckman spectrophotometer. For wave lengths below 474 mμ., 2·0 ml. stock solution was diluted to 7·0 ml. with control solution (25 % pyridine, 0·05 N-NaOH) and in this way maximum density values above 0·7 were avoided. This solution shows only a single band at 587, but when left standing for several hours in the presence of cysteine, was found to undergo change in the same way as the haem obtained from *C. diphtheriae* cells did. The 587 mμ. band faded and was replaced by two bands at 553 and 525 mμ.

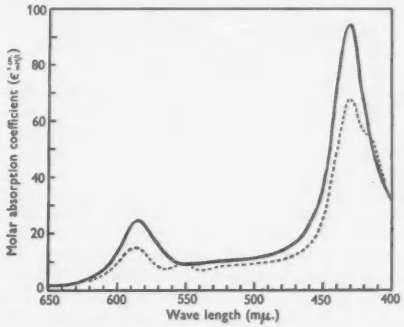


Fig. 4. Absorption spectra of pyridine haemochromogens. Heart haemochromogen *a* ——; *C. diphtheriae* haemochromogen *a* - - - - -. Solvent, 3·11 M-pyridine in 0·05 N-NaOH.

Removal of iron. The method of Warburg & Negelein (1932) was used for the preparation of the porphyrin. In Table 5 are presented the absorption maxima and orders of intensities of the pigment in various solvents. Neutral organic solutions were coloured reddish when the porphyrin concentration was high and yellowish green in dilute solution. The porphyrin was insoluble in light petroleum (b.p. 40–60°).

From the data presented it was concluded that haem *a* isolated from ox-heart muscle was identical with that from *C. diphtheriae* cells, but, fortunately,

Table 4. *Absorption spectra of haem* a *derivatives*

Compound	Solution	Absorption bands: I (mμ.)	II (mμ.)	Soret (mμ.)	Order of intensity
Haem *a*	KOH (0·17 N)	635	577	—	I, II
Haem *a*	Alkali + $Na_2S_2O_4$	578	531	—	I, II
CO-haem *a*	Alkali + $Na_2S_2O_4$ + CO	591	542	—	I, II
Pyridine haemochromogen *a*	Alkali + pyridine + $Na_2S_2O_4$	587	—	430	—

Table 5. *Absorption bands of ox heart dichroic porphyrin* a*

Solvent	I (mμ.)	II (mμ.)	III (mμ.)	IV (mμ.)	E.A.† (mμ.)	Order of intensity
$CHCl_3$	648	590	561	520	450	III, II, IV, I
Benzene	647	591	561	519	451	III II, IV, I
Glacial acetic acid	641	591	560	520	454	III, II, IV, I
Ether	642	587	556	515	439	III, II, IV, I
Pyridine	648	589	559	527	444	III, II, IV, I
Pyridine-ether	647	589	558	517	455	III, II, IV, I
HCl (10 g./100 ml.)	618	563				II, I
KOH (0·1 N)	582	565	519		447	II, III, I,

* From measurements with the Beck-Hartridge spectrometer.
† End absorption.

the former contained none of the secondary product seen in the material isolated from the micro-organisms.

Experiments to confirm the suitability of isolation procedures

In order to test the possibility that the dichroic pigment might have been an artifact derived from protohaem, similar processes of extraction and separation were performed on aqueous acetone solutions (acid, neutral and alkaline) of pure protohaemin. After these solutions had been allowed to stand for 2 days at room temperature, with frequent shaking in air, no haem component was detected with characteristics similar to the above dichroic pigment. Whole ox blood, when subjected to the processes, did not yield any pigment with preference for the ether phase.

Further experiments with haem a

The more readily obtainable product from ox-heart muscle was used in these investigations.

Effect of carbon monoxide on haem a. On bubbling CO through an alkaline solution of haem *a* reduced with $Na_2S_2O_4$, there was an immediate shift of the absorption spectrum to give bands at 591 and 541·5 mμ.

Effect of hydroxylamine on haem a. A solution of Na_2CO_3 was added to a pyridine solution of the haem to give a 20% (v/v) final concentration of pyridine. The addition of a few crystals of hydroxylamine hydrochloride followed a few minutes later by the addition of $Na_2S_2O_4$ resulted in the appearance of a haemochromogen spectrum with maxima at 570 and 533 mμ. (cf. haemochromogen spectrum of original haem 587 mμ.).

Further experiments on the porphyrin obtained from haem a

Effect of cysteine on porphyrin a. The addition of cysteine hydrochloride to a warm pyridine solution of the porphyrin, containing anhydrous Na_2CO_3, resulted in a shift of band positions to I, 634; II, 578; III, 546; IV, 508 mμ. (order of intensity: III, IV, II, I).

Effect of hydroxylamine on porphyrin a. The porphyrin was dissolved in pyridine and anhydrous Na_2CO_3 added. The addition of a few mg. of hydroxylamine hydrochloride was followed by an immediate shift of band positions, even at room temperature, and after this change no further shift was observed by heating at 100° for 1 hr. The mixture was diluted with water and shaken with ether. The pigmented ether phase was washed well with 1% (w/v) HCl and finally with water to remove traces of acid. The spectrum in washed ether was I, 639; II, 582; III, 547; IV, 509 mμ. End absorption (E.A.): 444 mμ. (order of intensity: III, IV, II, I). The addition of an equal volume of pyridine gave the following bands: I, 637; II, 582; III, 549; IV, 509 mμ.

In Fig. 5 this oxime reaction at 15° is demonstrated by measurements made in the Beckman spectrophotometer. The solutions used to obtain the curves were identical except for the addition to one of a small amount of hydroxylamine hydrochloride.

Copper complexes of porphyrin a. The copper complex was formed by adding a small amount of cupric acetate to a solution of the free porphyrin in glacial acetic acid and heating in a boiling water bath for 5 min. The absence of red fluorescence under ultraviolet radiation indicated completion of the reaction. In Table 6 are shown the positions of absorption band maxima in various solvents.

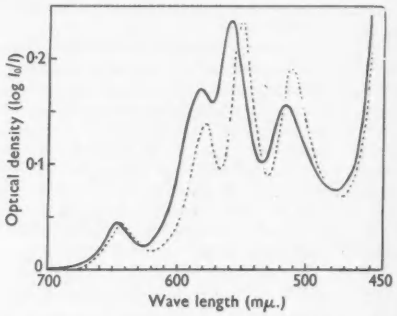


Fig. 5. Absorption spectra of heart porphyrin *a* derivatives. Heart porphyrin *a* ——, heart porphyrin *a* oxime - - - -. Solvent pyridine.

Table 6. *Absorption bands of copper complex of heart dichroic porphyrin* a

Solvent	I (mμ.)	II (mμ.)	III (mμ.)	Order of intensity
Glacial acetic acid	597	552	—	I, II
Pyridine	599	553	—	I, II
Ether	594	548	525	I, II, III
Pyridine-ether	597	552	530	I, II, III

The addition of hydroxylamine hydrochloride to a pyridine solution of the copper complex containing anhydrous Na_2CO_3 brought about a shift of band positions within a few minutes at room temperature, to I, 587; II, 542; III, *504* mμ. E.A.: 442 mμ. (order of intensity: I, II, III).

Esterification of porphyrin a. The porphyrin *a* was allowed to stand 2 days at 4° in methanol saturated with HCl; absorption bands I, 605; II, 563 mμ. (order of intensity: II, I). The pigment was taken into $CHCl_3$ after dilution with water. The $CHCl_3$ solution was extracted 4 times with 2 N-NH_4OH, 3 times with 10% (w/v) NaCl and then run through a dry filter paper. After evaporation to dryness and solution of the residue in pyridine-ether solution, bands were observed at 648, *630*, 589, 560, 510 mμ. The presence of the band at 630 mμ. and the general appearance of the band pattern indicated that a small amount of the pigment had undergone change. After extraction of this solution with 10% (w/v) HCl to remove pyridine and traces of modified porphyrin the absorption bands were measured again after washing with water and dilution with an equal volume of pyridine (646, 589, 558, 516 mμ.) cf. Table 4. The treatment of porphyrin *a* (dissolved in ether) with diazomethane did not appear to produce any modified pigment and so was considered preferable to the method described above for esterification.

DISCUSSION

The spectrometric tests made on intact cell suspensions of *C. diphtheriae* demonstrate the effect of iron concentration upon cytochrome content. The faint

absorption bands seen in washed cells grown at the lower iron levels agreed in wave length with the three main bands of cells grown at higher levels. It is concluded that, although the main types of haem constituents bound within the cells are independent of iron concentration, the quantity produced is decreased by low concentrations of iron and such inhibition is associated with an increased excretion of coproporphyrin III. A five- to ten-fold increase of intracellular haem components seems to take place when cells are grown at iron levels sufficient to reduce toxin and coproporphyrin production in the medium from optimal to insignificant amounts.

The absorption patterns and mean positions of wave lengths of maxima seen in reduced cell suspensions demonstrated the presence of cytochrome components. The intense band at 562 mμ. and the band of lower intensity at 531 mμ. resemble the α and β bands of cytochrome *b* except that they are shifted to values slightly lower than the *b* bands of some other tissues. Other micro-organisms show similarly low values. Small differences in band positions measured by direct vision spectroscopy, may not always be significant, and confirmatory specific functional tests are needed before they are attributed to new components.

The absence of cytochrome *c* was apparent by examination of the 562 mμ. band. The general appearance of this band with its edges at 568 and 554 mμ. makes it difficult to assume any contribution from the 550 mμ. α-band of cytochrome *c*. This was further confirmed by comparison of *C. diphtheriae* cells with other micro-organisms known to possess cytochrome *c*. The absorption band at 600 mμ. indicates the presence of the cytochrome *a* complex. Experiments similar to those used in general cytochrome studies allowed us to confirm the presence of cytochrome oxidase (Pappenheimer, 1947*b*).

The shift of absorption bands to shorter wave lengths on the addition of pyridine to reduced cell suspensions results from a displacement, by this base, of the normal protein moieties of the cytochrome. The 587 mμ. band represents the pyridine haemochromogen complex of the *a* component and the bands at 557 and 526 mμ. that of the prosthetic group of cytochrome *b*. The positions of the latter bands coincide with the α and β bands of pyridine protohaemochromogen. Pyridine coprohaemochromogen exhibits bands at 548 and 518 mμ., and as these cannot be demonstrated in 'high iron' cells the iron complex of coproporphyrin III must be absent. It was concluded from the above tests that at least two cytochrome components were present (viz. *a* and *b*) with dissimilar prosthetic groups. The identification of the major component as protohaem no. 9 is confirmed by all tests on the crystalline product isolated from the aqueous phase. Quantitative measurements on acid-acetone extracts, after conversion of the pigment into pyridine haemochromogen or protoporphyrin, gave values equivalent to 1·0–1·1 mg. protohaem/l. of culture. As the 587 mμ. pyridine haemochromogen band of component *a* (see p. 254) is known to possess a similar extinction value to protohaemochromogen, its low intensity compared with that of the 557 mμ. absorption band of the latter component in intact cells must mean that it represents a small fraction of the total intracellular haems.

The dichroic pigment in the ether phase (from *C. diphtheriae* cells) was composed of two haems. The following facts point to derivation from one original component: both haems showed preference for the ether phase, the porphyrins derived from them both possessed high acid numbers, and on standing in solution the component with haemochromogen bands at 553 and 525 mμ. increased at the expense of the predominant 587 mμ. component. The reactions with hydroxylamine and cysteine also supported this conclusion.

As the interpretations of data were complicated by the presence of a product derived from the original haem it was decided that further studies to ascertain the constitution of the pigment might be facilitated by preparation of material from a more convenient source than *C. diphtheriae* cells. The haem *a* from ox-heart muscle possessed spectroscopic characteristics similar to those of the haem from *C. diphtheriae* cells and (presumably due to smaller amounts of reactive impurities) was far more stable during its separation, and was therefore the material of choice. Lipid material accompanied the haem so closely during extraction that the possibility that some of it is an integral constituent cannot be excluded (cf. chlorophylls *a* and *b*).

In experiments with heart muscle our primary aim was to study the dichroic haem *a*. One pigment with haemochromogen α and β bands at wave lengths slightly shorter than protohaemochromogen was found in small amounts in the aqueous acid-acetone phase after extraction with ether. Further work is in progress to establish the nature and significance of this pigment. Although cytochrome *c* is not dissociated by treatment with acetone and hydrochloric acid it is not impossible that some pigment is dissolved. The great affinity of the *c* component for aqueous phases could then explain the presence of a haem derivative in the acid-acetone layer.

Although it had been concluded that the pigment responsible for the 587 and 430 mμ. absorption bands, in pyridine extracts of *C. diphtheriae* cells and ox-heart muscle, was the prosthetic group of the cytochrome *a* component it was necessary to prove that this haem had not arisen as a degradation product of protohaem. Attempts to produce degradation products of protohaem under the conditions applicable during extraction procedures failed (cf. Negelein,

1932*a*, *b*; Negelein, 1933; Roche & Benevent, 1936). The claim was made in the paper last mentioned that another derivative, obtained from heart muscle by a different process and possessing pyridine haemochromogen bands at 587, approx. 554, 530 and 425 mμ., was, in fact, the true haem *a* from cytochrome *a*. An inspection of the absorption curve of this pigment, however, leads us to suggest that the product of Roche & Benevent (1936) was a mixture composed of one component with a band at 587 mμ. and another component with bands at approx. 554 and 530 mμ., similar to the haemochromogen of the pigment found to accompany haem *a* in preparations from *C. diphtheriae* cells. For reasons stated earlier we consider this material (maxima 553, 525 mμ.) to have been derived from the haem *a* by combination with substances accompanying the latter during extraction (compare the action of cysteine on haem *a*). The stability of our haem *a* to acid offers another distinct contrast with the product described by these workers. The 'kryptoporphyrin' obtained by the transformation of protoporphyrin (Negelein, 1932*b*) possessed a lower acid number than the dichroic pigment isolated by us from *C. diphtheriae* cells and heart muscle.

The α-band position of the carbon monoxide-haem *a* complex (591 mμ.) is reminiscent of the position of one band of the light-sensitive carbon monoxide compound of Warburg's respiratory enzyme (cytochrome oxidase of Keilin). At the present time it is considered that cytochrome *a* and cytochrome oxidase are related at least in the nature of their prosthetic groups.

The quantitative absorption curve of pyridine haemochromogen *a* (Fig. 4) shows that it differs considerably from the normal type of haemochromogen. In general, α and β bands are observed in the visible region of the spectrum, and even in the case of chlorocruorin haemochromogen we find, in addition to an α band at 583 mμ., a second, weaker band at 542 mμ. The extinction coefficients of the band maxima at 587 and 430 mμ. of haemochromogen *a* approach those of the majority of haemochromogens, but the greater width of the former band makes it difficult to appreciate in direct vision spectroscopy that such is the fact.

The effect of cysteine on the pyridine haemochromogen *a* is similar to that reported for many haem derivatives (Fischer & Mittermair, 1941). Fischer & Mittermair concluded from experiments with derivatives of chlorophyll *b* that the shift was due to the presence of a reactive aldehyde group which with cysteine gave compounds of the mercaptal acetal type. The experiments we have reported with hydroxylamine and cysteine on both haem *a* and its porphyrin therefore indicate the presence of a carbonyl group in our material. There is the possibility that the carbonyl groups are ketonic, but the rapidity of the addition reactions suggests that labile aldehyde groups are involved.

The reaction of haem *a* with hydroxylamine results in the formation of an oxime, the pyridine haemochromogen bands of which are situated at 570 and 533 mμ. In the case of chlorocruorin haemochromogen (1:3:5:8-tetramethyl-2-formyl-4-vinylporphin-6:7-dipropionic acid iron complex), oxime formation shifts its 583 mμ. band to 557 mμ., i.e. identical with the haemochromogen of protoporphyrin no. 9, and, on this fact, it is assumed that the $-CH=NOH$ group exerts an effect similar to that of a $-CH=CH_2$ group (compare the haemochromogens of mesoporphyrin and protoporphyrin). The shift of band position shown by haemochromogen *a* on conversion to oxime is 17 mμ., a value which is similar to the shift (16·5 mμ.) shown by formylpyrroporphyrin no. 15, but much lower than in the case of chlorocruorin haemochromogen (26 mμ). This comparison indicates that haemin *a* must possess structural groups (other than such active carbonyl groups) either different in type or in their arrangement around the nucleus from those found in protohaemin or chlorocruorohaemin.

The method used for preparing porphyrin *a* appeared to involve no significant modification of substituent groups, as the re-introduction of iron produced the same type of haemochromogen spectrum as that of the original substance. We could not find any evidence that the reaction with hydroxylamine proceeds in two stages, and so it must be assumed that in porphyrin *a* there is only one labile aldehyde group/mol. of pigment. The curves of Fig. 5 illustrate the differences between the oxime and original porphyrin *a*. All bands are shifted towards the blue, bands I and III remain unchanged in extinction values, but bands II and IV reverse their relative intensities. The oxime shows the band relationships (III, IV, II, I) characteristic of the 'rhodo-type' of porphyrin.

SUMMARY

1. The cells of *Corynebacterium diphtheriae*, when grown at iron levels sufficient to inhibit the excretion of toxin and coproporphyrin, showed a five- to ten-fold increase of haem components over the level present when toxin and porphyrin excretion are maximal. The presence of cytochrome oxidase (cytochrome *a* complex) and cytochrome *b* were confirmed spectroscopically.

2. Methods for the separation of the two main haem prosthetic groups are described. Crystalline protohaemin, which is assumed to be derived at least in part from cytochrome *b*, and a dichroic haem, presumably from the cytochrome *a* complex, were obtained.

3. The dichroic haem was identified with the

haem of cytochrome *a* of ox-heart muscle and further studies on its characteristics were made on material from this more accessible source.

4. The general properties of this haem and its reactions with pyridine, cysteine, carbon monoxide and hydroxylamine were investigated.

5. The free porphyrin, like the haem from cytochrome *a*, reacted with cysteine and hydroxylamine.

6. The results of the tests described indicate that this haem possesses at least one aldehyde group.

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The Oxidation of Manganese by Plant Extracts in the Presence of Hydrogen Peroxide

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Manganese is known to be an essential micronutrient of plants, although its function is unknown. It apparently plays a part in plant respiration; Lundegårdh (1939) found that the oxygen uptake of manganese-deficient wheat roots was raised by 155–470% by the addition of 5×10^{-5}M manganese chloride. Such an effect might be brought about by the activation of certain enzyme systems, e.g. arginase (Waldschmidt-Leitz & Purr, 1931), phosphoglucomutase (Cori, Colowick & Cori, 1938), leucylpeptidase (Berger & Johnson, 1939) or by a system in which Mn^{++} undergoes alternate oxidation and reduction. It has been shown that soil micro-organisms can oxidize Mn^{++} (e.g. Beijerinck, 1913; Gerretsen, 1937; Leeper & Swaby, 1940; Mann & Quastel, 1946). No satisfactory evidence has been put forward to show that Mn^{++} is oxidized in higher plants and the present work was undertaken to investigate whether such oxidation does take place.

MATERIALS AND METHODS

Preparation of plant extracts. The roots were scrubbed free from soil and minced twice in a meat mincer. The mince was weighed and the juice squeezed out by hand through madapollam. Water to one-quarter of the original weight of the mince was added to the residue, which was then ground in a mortar with sand and squeezed through madapollam. This treatment was repeated twice. The extracts were combined and filtered through Whatman no. 1 filter paper, and stored in a refrigerator. Most of the work was done with horse-radish (*Cochlearia armoracia*); it was found that extracts of this root showed little loss of activity over a period of several weeks. In view of the possibility of contamination, however, fresh extracts were frequently made. Extracts of other roots were made and used on the same day. This method was originally adopted with horse-radish as benzidine-H_2O_2 tests showed satisfactory extraction of peroxidase.

Catalase preparation. The caps of the Basidiomycete, *Marasmius oreades*, were ground with sand, squeezed through madapollam and centrifuged at 3500 rev./min.

(1500 *g.*) for 0·5 hr. The catalase activity (Katalasefähigkeit) of the supernatant liquid, estimated by the method of Sumner & Somers (1943), was about 80. The catalase activity diminished rapidly and consequently the preparation was made and used on the same day.

Peroxidase preparation. The peroxidase preparation was made by the method of Keilin & Mann (1937) from horse-radish. After precipitation with $(NH_4)_2SO_4$, followed by fractional precipitation with ethanol and further purification with tricalcium phosphate gel, a preparation of Purpurogallinzahl (P.Z.) 290 was obtained. The P.Z., i.e. mg. purpurogallin formed by 1 mg. enzyme preparation in 5 min. from pyrogallol and H_2O_2 under fixed conditions, was determined by the method of Keilin & Mann (1937).

Preparation of hydrated manganese dioxide. The suspension of hydrated MnO_2 was prepared by the method of Heintze & Mann (1949), which is based upon the dismutation of manganipyrophosphate in alkaline solution. The valency, as determined from the Mn content of the preparation and its oxidizing capacity towards oxalic acid, was 4·08.

Manometric measurements were carried out in the Warburg apparatus at 25°. The volume of the reaction mixture was 3 ml. and KOH was present in the centre cup unless otherwise stated.

EXPERIMENTAL AND RESULTS

Colorimetric evidence of manganese oxidation

In preliminary experiments with certain types of root extracts colour reactions were obtained which indicated the formation of manganic complexes. The addition of $MnSO_4$ and H_2O_2 to the extracts in the presence of citrate at pH 7 caused the development of a greenish yellow colour, while in pyrophosphate at this pH a pink colour was obtained. On acidification of the pyrophosphate solution the colour changed to a brighter pink; both the pink and greenish colours were rapidly discharged by the addition of acid H_2O_2 or hydrazine sulphate. These reactions are typical of manganipyrophosphate and citrate complexes. The colour reactions were not given by extracts which had been heated for 5 min. in a boiling water bath, nor were they given in absence of H_2O_2 or Mn^{++}.

Reaction mixtures consisting of 0·4 ml. root extract, 0·2 ml. 0·1 M-$MnSO_4$, 2 ml. 0·1 M-citrate (pH 7) or 2 ml. 0·5 M-pyrophosphate (pH 7) and 0·2 ml. water were made up in test tubes and 0·2 ml. 0·05 M-H_2O_2 added. The rate of development and the intensity of the pink or greenish yellow colour was followed for a 15 min. period. The strongest reactions were given by extracts from horse-radish (*Cochlearia armoracia*) and turnip (*Brassica campestris* L.), with which the colour developed within a minute of adding H_2O_2. Weaker reactions were given by extracts of carrot peel (*Daucus carota*) and spinach beet (*Beta vulgaris*); dandelion (*Taraxacum officinale*) extracts were inactive. Difficulty was experienced with some extracts, e.g. potato which, owing to the presence of strong direct oxidase systems, rapidly became so dark as to obscure any colour due to manganic complex formation.

Isolation of manganese dioxide

In neutral or acid pyrophosphate, MnO_2 dissolves on the addition of $MnSO_4$ to give a pink manganipyrophosphate complex (Heintze & Mann, 1946, 1949). At pH values 'much above 8' the complex dismutes into hydrated MnO_2 and manganopyrophosphate (Lingane & Karplus, 1946):

$$Mn_2O_3 \rightleftharpoons MnO + MnO_2.$$

By making use of the dismutation in alkaline solution an attempt was made to prove that the colour formation with root extracts and H_2O_2 was due to the formation of manganic complexes.

To a reaction mixture containing 455 ml. 0·5 M-pyrophosphate buffer (pH 7), 25 ml. horse-radish extract and 20 ml. 0·5 M-$MnSO_4$, H_2O_2 (5 ml., 0·05 M) were added at 10 min. intervals, with stirring, until the solution became a deep red and further addition of H_2O_2 did not appear to intensify the colour. In all, 25 or 30 ml. of 0·05 M-H_2O_2 were added; 20 min. after the last addition, 0·15 ml. catalase preparation was added. (No H_2O_2 could be detected in the solution at this stage. It was necessary, however, to add the catalase preparation to control (*B*) and consequently the reaction mixture was similarly treated.) After a further 20 min. the mixture was adjusted to pH 10 (glass electrode) by the addition of 2 N-NaOH. During the addition of NaOH a brisk current of N_2 was blown through the solution to assist mixing and exclude atmospheric O_2, thus preventing autoxidation of the remaining $MnSO_4$ due to local excess of alkali.

The mixture, which was now a dark brown, was allowed to stand for 3 hr. to ensure complete dismutation and then centrifuged. The brown sediment was washed on the centrifuge 4 times with 50 ml. 0·2 M-pyrophosphate (pH 9·8), 3 times with 50 ml. 0·2 M-pyrophosphate (pH 7·0) and finally twice with 50 ml. water, to remove Mn^{++} which is absorbed by hydrated MnO_2. The sediment was ground thoroughly

Table 1. *Valency of manganese in oxidation product*

(Mn content was estimated colorimetrically; oxidizing capacity by manometric measurement of O_2 output: 0·5 ml. suspension, 2·3 ml. N-H_2SO_4 in main vessel and 0·2 ml. 0·5 M-H_2O_2 in 0·1 N-H_2SO_4 tipped from side arm.)

Preparation no.	Total 0·05 M-H_2O_2 added in preparation (ml.)	Manganese (mg./ml. of suspension)	Oxygen (μl. O_2/ml. of suspension)	Valency	Total MnO_2* isolated (mg.)	Yield of MnO_2 based on total H_2O_2 added (%)
1	25	0·775	304	3·92	29·5	27
2	30	1·254	518	4·03	51·8	40

* Calculated from oxidizing capacity.

in a mortar and made up in water to 25 ml. Portions were taken for colorimetric determination of manganese and the oxidizing capacity was estimated manometrically with acid H_2O_2. Two preparations were made: the valency of the manganese was found to be 3·92 in one case and 4·03 in the other (Table 1).

Two control experiments were made, one in which no H_2O_2 was added (*A*), and the other in which horse-radish extract previously heated for 10 min. at 100° (*B*); these mixtures were worked up similarly to the reaction mixture, except that no catalase was added to control (*A*). Immediately after the addition of catalase to (*B*), a sample was transferred to a Warburg vessel and the evolution of O_2 was followed. This was complete in 25 min. after which 0·2 ml. MnO_2 suspension (2·6 mg. MnO_2/ml.) was tipped from the side arm. No further evolution of O_2 took place showing that the H_2O_2 had been completely decomposed by the catalase. After adjustment to pH 10 and standing for 3 hr. the control (*B*) was yellow brown and control (*A*) yellow, indicating the presence of a small amount of colloidal MnO_2. The colour was, however, less brown than the supernatant obtained by centrifuging the reaction mixture at this stage and no sediment was obtained on centrifuging.

It was clear from the experiments that the oxidation depended on the presence of H_2O_2 and a thermolabile factor in the plant extract.

Evidence for the oxidation of manganese based on the accumulation of manganipyrophosphate

It is well known that MnO_2 decomposes H_2O_2 with evolution of O_2; in water the reaction is catalytic, but in acid solution reduction of MnO_2 may take place and under suitable conditions of acidity (e.g. in 0·1 N-H_2SO_4) the reaction is stoicheiometric,

$$MnO_2 + H_2O_2 = MnO + O_2 + H_2O.$$

Experiments were carried out to find out whether the decomposition of H_2O_2 by manganipyrophosphate was catalytic or stoicheiometric under the conditions used. The behaviour of hydrated MnO_2 and $MnSO_4$ under the same conditions was also investigated.

Decomposition of H_2O_2 by MnO_2 and manganipyrophosphate. Known amounts of MnO_2, or manganipyrophosphate and H_2O_2 were allowed to react and the output of O_2 measured manometrically (Table 2). In water the H_2O_2 was decomposed catalytically by MnO_2, but in the other media the reaction was generally stoicheiometric. In orthophosphate at pH 7 the decomposition was somewhat larger than stoicheiometric, but there was also more decomposition in the control. With manganipyrophosphate the reaction was stoicheiometric. The spontaneous decomposition of H_2O_2 was slight, and not significantly affected by the presence of 110 μg. Mn^{++}.

Conditions leading to the accumulation of manganipyrophosphate. Since manganipyrophosphate reacts stoicheiometrically with H_2O_2 its accumulation as an oxidation product must depend on its rate of formation being greater than its rate of reduction by H_2O_2, but during the oxidation of Mn^{++} by horse-radish root extract and H_2O_2 in presence of pyrophosphate an evolution of oxygen would be expected owing to reduction of part or all of the manganipyrophosphate by the H_2O_2. If there is no accumulation of manganipyrophosphate and no side reaction the total O_2 evolved should be equivalent to the H_2O_2 initially present. If manganipyrophosphate accumulates the total oxygen output will be lower since part of the H_2O_2 is used up in this accumulation. These reactions can be followed manometrically as shown in Fig. 1.

Table 2. *The decomposition of H_2O_2 by MnO_2 and manganipyrophosphate*

(With MnO_2 the reaction mixtures consisted of 106 μg. MnO_2 (hydrated) and 0·0033 M-H_2O_2, in 0·033 M-buffer at pH 7 or in water, with or without the addition of horse-radish extract (0·4 ml., heated 10 min. at 100°). The manganipyrophosphate was preformed by the addition of 110 μg. Mn^{++} to 106 μg. MnO_2 in pyrophosphate at pH 7. The H_2O_2 was tipped from the side arm except when horse-radish extract was used when the MnO_2 was tipped. Control experiments were made with 110 μg. Mn^{++}. Theoretical O_2 output for complete catalytic decomposition of H_2O_2: 112 μl., in absence of catalysis, 24·4 μl.)

	Oxygen output (μl. O_2)				
Time (min.)	Water (MnO_2)	Ortho-phosphate (MnO_2)	Pyro-phosphate (MnO_2)	Horse-radish extract heated at 100° (MnO_2)	Manganipyro-phosphate
10	104·5	23·0	23·8	25·3	23·5
20	108·5	23·0	24·4	26·0	24·9
60	109·5	29·7	26·1	29·0	27·6
120	111·0	35·3	28·4	29·5	30·3
	Control with 110 μg. Mn^{++}				
120	9·0	12·0	3·0	3·0	3·0

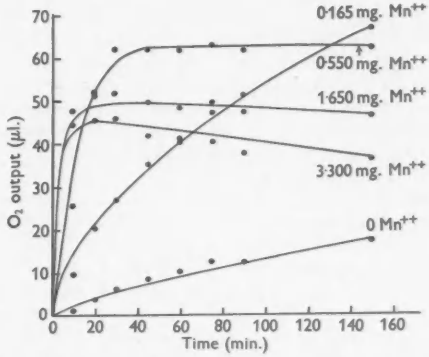


Fig. 1. The accumulation of manganipyrophosphate when horse-radish extract is treated with H_2O_2 in the presence of added Mn^{++}. The reduction in O_2 output at the higher levels of Mn^{++} indicates manganipyrophosphate accumulation; theoretical O_2 output: 112 μl.

In this experiment the reaction mixtures consisted of 0·4 ml. horse-radish extract in presence of 0·45 M-pyrophosphate at pH 7 and 0·2 ml. of 0·05 M-H_2O_2 tipped from the side arms, the Mn^{++} added being varied from 0 to 3·3 mg. The slow evolution of O_2 which occurred with the root extract alone was markedly increased on addition of Mn^{++}. The initial output was more rapid with the higher amounts of Mn^{++}, but the total output was greatest with the lowest amount of Mn^{++}. In absence of horse-radish extract the output was negligible. These results confirm the view that two reactions are proceeding, the oxidation of the added Mn^{++} by the root extract and H_2O_2, and the reduction of the oxidation product by H_2O_2 with evolution of O_2. That the lower total outputs with the higher concentrations of Mn^{++} were due to the accumulation of manganipyrophosphate was supported by the fact that the most intense pink colour was in the flask with the highest concentration of Mn^{++}.

The system may catalyse the oxidation of manganese to the manganic form or to MnO_2. In orthophosphate at pH 7 a stable manganic complex cannot be formed and breakdown will occur into MnO_2 and MnO, the final product being MnO_2. If a stable manganic complex can be formed, as in pyrophosphate, it will accumulate provided that the rate of formation is greater than that of reduction by the H_2O_2. Increasing the concentration of Mn^{++} would therefore favour accumulation by accelerating the rate of oxidation. The fact that Mn^{++} is much less soluble in orthophosphate (pH 7) than in pyrophosphate (pH 7) may partly explain why no accumulation of MnO_2 has been observed in orthophosphate.

Assuming that the primary oxidation product of Mn^{++} is MnO_2, the following reactions may take place in the pyrophosphate reaction mixture:

(1) $MnO \rightarrow MnO_2$;
(2) $MnO_2 + H_2O_2 \rightarrow MnO + O_2 + H_2O$;
(3) $MnO + MnO_2 \rightarrow Mn_2O_3$;
(4) $Mn_2O_3 + H_2O_2 \rightarrow 2MnO + O_2 + H_2O$.

If (2) is more rapid than (1) MnO_2 cannot accumulate, but under conditions where (3) is favoured at the expense of (2), i.e. at high Mn concentration, Mn_2O_3 will be formed, and will accumulate if (4) is slow in comparison with (3).

When hydrated MnO_2 was allowed to react with dilute H_2O_2 in pyrophosphate at pH 7, it was noticed that after the disappearance of the brown MnO_2 the reaction mixture turned pink, and in spite of the presence of excess H_2O_2 this colour faded only slowly. The intensity of this pink colour, which was presumably due to manganipyrophosphate, could be increased if $MnSO_4$ was added together with the H_2O_2 to the MnO_2. The formation of manganipyrophosphate in the experiment with horse-radish extracts may therefore be due to reaction (3), particularly when large additions of Mn^{++} are made initially.

Since, as is shown below (Fig. 2), reaction (2) is more rapid than (4), the conversion of MnO_2 to manganipyrophosphate favours the accumulation of oxidized Mn.

The reaction between hydrated MnO_2 and H_2O_2 in pyrophosphate at pH 7 is very rapid, particularly at high H_2O_2 concentration, and although that with manganipyrophosphate is slower it is difficult to demonstrate this manometrically.

The conditions finally chosen as satisfactory were as follows: 0·264 mg. MnO_2 (hydrated) in 0·16 M-pyrophosphate at pH 7 in one vessel, and 0·264 mg. MnO_2 (hydrated) plus 0·1 ml. 0·05 M-$MnSO_4$ (to give manganipyrophosphate) in the other vessel. KOH was omitted from the centre cup and the volume of the reaction mixtures was 5 ml. After equilibration, H_2O_2 to give a concentration of 0·0006 M was tipped into both vessels simultaneously by two operators, and the O_2 output was measured at 2 min. intervals.

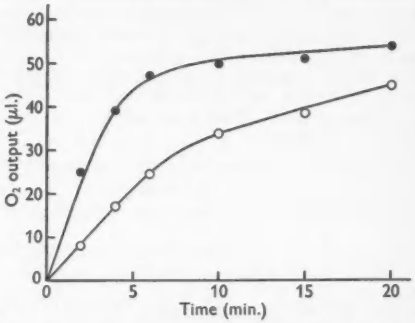


Fig. 2. A comparison of the velocity of the reactions between $MnO_2 + H_2O_2$ and manganipyrophosphate + H_2O_2; ●—●, $MnO_2 + H_2O_2$; ○—○, manganipyrophosphate + H_2O_2.

With MnO_2 most of the H_2O_2 was decomposed in the first 5 min. with a velocity 2 to 3 times that with manganipyrophosphate (Fig. 2). After 1 hr. the output was the same in both cases within experimental error. The comparison is not strictly between MnO_2 and manganipyrophosphate, since manganipyrophosphate would be formed as the reaction between MnO_2 and H_2O_2 proceeded. This probably explains the slow output in the later stages where MnO_2 was used. Furthermore, the hydrated MnO_2 was in the form of a suspension and the rate of the reaction would depend on the particle size, whereas the manganipyrophosphate was in solution.

Estimation of manganipyrophosphate by hydrazine. It is known that MnO_2 reacts quantitatively with hydrazine in the following way:

$$2MnO_2 + N_2H_4 \rightarrow N_2 + 2MnO + 2H_2O.$$

Manganipyrophosphate also reacts with hydrazine giving a gas output slightly higher than that required by theory (Table 3), and can be estimated in this way. Experiments in which the horse-radish extract was allowed to react with H_2O_2 in 0·67 M-pyrophosphate and in the presence of varying concentrations of $MnSO_4$ were carried out manometrically in an atmosphere of N_2. When the O_2 output ceased, hydrazine was tipped from the side arm, and a rapid evolution of gas, presumably N_2,

occurred. The output was greatest where the highest accumulation of manganipyrophosphate was expected and was accompanied by discharge of the pink colour. If the reactions in the first part of the experiment led only to the evolution of O_2 and the accumulation of manganipyrophosphate, then the combined O_2 and N_2 gas outputs should be equivalent to the H_2O_2 added. In experiments using 0·4 ml. horse-radish extract, the combined gas outputs were considerably lower than this, but were only 5–10% lower when 0·1 ml. extract was used (Table 4). The reduction in the amount of extract used led to a decrease in the accumulation of manganipyrophosphate. The fact that the output of N_2 was less than the theoretical suggests either that part of the H_2O_2 was lost in a side reaction, or that part of the MnO_2 or manganipyrophosphate formed was reduced by the extract. Reduction of MnO_2, and to a less extent of manganipyrophosphate, takes place when these compounds are incubated with the horse-radish extract (Table 3).

The properties of the manganese oxidizing system

It has been demonstrated in the previous experiments that horse-radish extracts in presence of H_2O_2 can bring about the oxidation of Mn^{++}. To identify and estimate the oxidation product it was necessary to carry out the experiments under conditions in which a stable manganic complex could accumulate, i.e. in pyrophosphate at pH 7 and with a high concentration of Mn^{++}. If the system is of physiological significance, however, oxidation should take place at low concentration of Mn^{++}. The oxidation product does not accumulate under these conditions because it reacts with H_2O_2 with evolution of O_2 as rapidly as it is formed. The oxidation can therefore be demonstrated manometrically.

The effect of small amounts of Mn^{++} *on the decomposition of* H_2O_2 *by horse-radish extract.* With most of the horse-radish root extracts, but not with other root extracts, an increase in the rate of decomposition of H_2O_2 occurred on addition of very small amounts of $MnSO_4$. The experiments were carried

Table 3. *Reaction of* MnO_2 *and manganipyrophosphate with hydrazine in presence and absence of horse-radish extract*

(The MnO_2 content of a suspension of hydrated MnO_2 was determined by oxalate titration. 0·4 ml. portions (0·984 mg. MnO_2) were suspended in 0·67 M-pyrophosphate buffer in Warburg vessels. The gas space was then filled with N_2. After equilibration 0·2 ml. saturated aqueous $N_2H_4.H_2SO_4$ was tipped from the side arm; the gas output was measured. Similar experiments were carried out with manganipyrophosphate formed by adding 0·2 ml. of 0·1 M-$MnSO_4$ to the reaction mixtures. Experiments were also made to determine the effect of additions of 0·4 ml. horse-radish extract on the estimation.)

Method of estimation	Reaction mixture	Gas output (μl.)	MnO_2 (mg.)	Recovery (%)
Oxalate titration	MnO_2	—	0·984	—
Acid H_2O_2 (manometric)	MnO_2	248·5	0·965	98·0
N_2H_4 (manometric)	MnO_2	128·0	0·994	101·0
	MnO_2 tipped into extract + N_2H_4	125	0·972	98·5
	MnO_2 + extract incubated 30 min.	116·0	0·901	91·5
	MnO_2 + extract incubated 60 min.	110·8	0·861	87·5
	Manganipyrophosphate	133·5	1·037	105·0
	Manganipyrophosphate + extract incubated 30 min.	131·0	1·02	103·5
	Manganipyrophosphate + extract incubated 60 min.	127·0	0·987	100·5

Table 4. *Accumulation of manganipyrophosphate*

(Reaction mixtures consisted of horse-radish extract and $MnSO_4$ as below in 0·67 M-pyrophosphate at pH 7; 0·2 ml. 0·05 M-H_2O_2 was tipped from the side arm and the O_2 output was measured. When the O_2 output stopped 0·2 ml. saturated aqueous $N_2H_4.H_2SO_4$ was tipped from the second side arm and the N_2 output measured. Gas space N_2.)

	Extract *A* (0·4 ml.)			Extract *A* (0·1 ml.)		Extract *B* (0·2 ml.)				
$MnSO_4$ added (mg. Mn)	0·55	1·65	2·75	1·65	2·75	0·55	1·375	2·75	5·50	8·25
(1) O_2 output (μl.)	66·2	49·0	49·5	85·8	79·3	76·2	75·6	71·0	62·5	55·3
(2) N_2 output (μl.)*	25·1	43·6	48·5	25·7	27·9	22·9	30·2	36·2	41·8	46·8
(3) $O_2 + N_2$ output (μl.)	91·3	92·6	98·0	111·5	107·2	99·1	105·8	107·2	104·3	100·3
(4) Theory for $O_2 + N_2$ output (μl.)†	116·0	116·0	116·0	116·0	116·0	112·8	112·8	112·8	112·8	112·8

* Uncorrected for 105% recovery (see Table 3).
† Half O_2 output obtained by tipping the H_2O_2 into excess hydrated MnO_2 in N-H_2SO_4.

out manometrically with 0·4 ml. root extract in presence and absence of 0·033 M-ortho- or pyro-phosphate buffer, 0·2 ml. of 0·05 M-H_2O_2 being tipped from the side arm after equilibration. The results of a set of experiments in the absence of buffer are given in Fig. 3. In absence of added Mn^{++} a slow decomposition of H_2O_2 occurred. This was in general increased by the addition of 2·2 μg. Mn^{++}, and increased progressively with increasing amounts of Mn^{++}. In general, the experiments were carried out in air, but similar results were obtained when the gas space was filled with N_2, showing that direct oxidases were not concerned in the reaction.

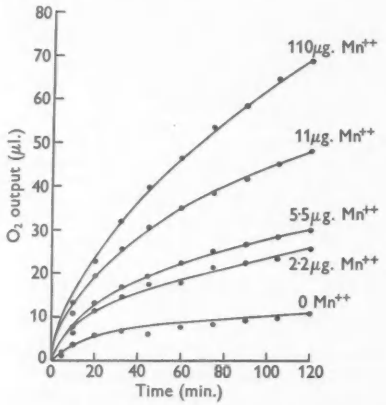


Fig. 3. The effect of added Mn^{++} on the rate of decomposition of H_2O_2 by horse-radish extract.

In ortho- and pyro-phosphate at pH 7 the rate of decomposition of H_2O_2 by the extract was higher than in the unbuffered extract (pH 4–4·5), but a similar effect of Mn^{++} was found. In orthophosphate at pH 7 with 11 μg. Mn^{++} added the output stopped in 80 min., the total output was 70 μl. O_2, about 63% of that required for complete decomposition of the H_2O_2 added (112 μl. O_2). No residual H_2O_2 could be detected in the reaction mixture and some H_2O_2 must therefore be lost in side reactions. In orthophosphate at pH 5 the rate of decomposition of H_2O_2, both in presence and absence of added Mn^{++}, was less than at pH 7. In general 0·4 ml. of extract produced the effects described above; with smaller amounts the response to added Mn^{++} was less, and usually with 0·05 ml. of extract little or no increase in the rate of decomposition of H_2O_2 was observed with additions of 110 μg. Mn^{++}. Some extracts gave little response to additions of 11 μg. Mn^{++} even when 0·4 ml. was used.

Control experiments (Table 3) showed that in all the media used the decomposition of H_2O_2 in absence of extract was not appreciably affected by addition of Mn^{++} and was not sufficiently great to affect the results of the above experiments.

The rate of decomposition of H_2O_2 by the horse-radish extract in different media with and without added Mn^{++} was reduced by previous heating at 100°. The effect of heat was somewhat variable and even after heating for 10 min. a slight activity remained in some cases.

It has already been shown that when large amounts of Mn^{++} are added to horse-radish extract and H_2O_2 the manganese is oxidized and the oxidation product reacts with H_2O_2 with evolution of O_2. It is considered therefore that the increase in rate of decomposition of H_2O_2 with small amounts of Mn^{++} is likewise due to oxidation of manganese. Since under the conditions of the experiments the reaction of MnO_2 and manganipyrophosphate with H_2O_2 is stoicheiometric (Table 2) it is obvious that if small amounts of Mn^{++} cause a large increase in O_2 evolution the Mn^{++} must undergo a cycle of oxidation and reduction.

The effect of various metals on the decomposition of H_2O_2 *by horse-radish extract.* The effect of Mn^{++}, Co^{++}, Cu^{++}, Zn^{++}, Ni^{++}, Fe^{++} and Fe^{+++} on the decomposition of H_2O_2 by the horse-radish extract was tested. At the concentration used (22 μg./3 ml.) only Mn^{++} produced an increase in the rate of decomposition.

Effect of variation in H_2O_2 *concentration.* The effect of varying the H_2O_2 concentration on the decomposition of H_2O_2 by horse-radish extract with 22 μg. added Mn^{++} in 0·033 M-orthophosphate at pH 7 was tested. The system was active over the range 0·02–0·00083 M-H_2O_2. With the higher concentrations an inhibitory effect was apparent in time. There was no reduction in the initial velocity, even with the lowest concentration.

Activation by an ether-soluble factor. The response of relatively inactive extracts, previously mentioned

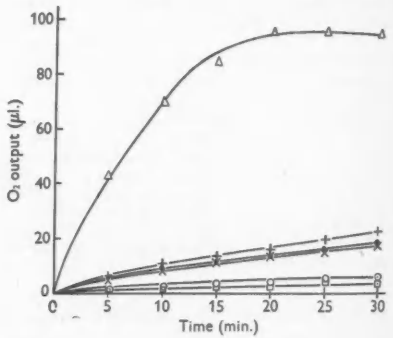


Fig. 4. The activation of horse-radish extract + Mn^{++} by an ether-soluble factor in the extract; ●—●, extract; +—+, extract + 55 μg. Mn^{++}; △—△, extract + ether-soluble factor + 55 μg.-Mn^{++}; ×—×, extract + ether-soluble factor; ⊡—⊡, ether-soluble factor + 55 μg. Mn^{++}; ⊙—⊙, 55 μg. Mn^{++}.

(p. 260), was increased considerably by the addition of an ether extract of acidified active horse-radish extract. The extract used was made from a residue from a peroxidase preparation and had previously been saturated with $(NH_4)_2SO_4$ and filtered; 200 ml. was acidified to pH 3 with N-H_2SO_4 and shaken

3 times with 30 ml. ether; the combined ether extracts were taken to dryness at room temperature and the residue suspended in 5 ml. water. The effect of 0·5 ml. of this suspension on the rate of decomposition of H_2O_2 by horse-radish extract which gave little response to 55 μg. Mn^{++} was tested in 0·033 M-orthophosphate at pH 7 in the presence and absence of Mn^{++} (Fig. 4). While the ether-soluble factor or Mn^{++} alone caused little or no increase in the activity of the extract, together they gave a marked increase. The ether-soluble factor was unaffected by heating for 15 min. in a boiling water bath, but was destroyed by ashing. It was clear therefore that a thermostable factor was a component of the manganese oxidation system.

The oxidation of manganese by a peroxidase system

The fact that H_2O_2 was necessary for the oxidation suggested that the thermolabile factor was a peroxidase. It was found that, on precipitation with $(NH_4)_2SO_4$ and dialysis, peroxidase preparations, obtained by the method of Keilin & Mann (1937), gave no evidence of manganese oxidation by colorimetric tests. Positive tests, however, were obtained with these and more highly purified peroxidase preparations, when the ether-soluble factor, previously described, or certain peroxidase substrates, such as *p*-cresol, were added. It was noted that whereas in the absence of manganese, *p*-cresol was oxidized by the peroxidase and H_2O_2 to give an insoluble white oxidation product, in the presence of Mn^{++} the solution remained clear and the coloured manganic complex appeared. These results suggested that the ether-soluble factor of the horse-radish extracts is a substrate for peroxidase and that manganese reduces the oxidized substrate and is thereby itself oxidized.

The oxidation of manganese by systems containing peroxidase can be followed by manometric measurement of the O_2 evolved. In the experiments shown in Fig. 5 the oxidizing system was: in the main vessel, 7 μg. of a peroxidase preparation with P.Z. 290, 0·00033 M-*p*-cresol in 0·033 M-orthophosphate at pH 7 to which varying additions (5·5–33 μg.) of Mn^{++} were made; 0·2 ml. 0·05 M-H_2O_2 was tipped from the side arm after equilibration. In absence of added Mn^{++} no evolution of oxygen took place, but with increasing amounts of Mn^{++} increases in the velocity and in the total output were observed. The theoretical output (112 μl. O_2) was obtained with 33 μg. added Mn^{++}.

In the reaction mixture without Mn^{++}, a white precipitate was present, indicating that the *p*-cresol had been oxidized. This precipitate was smaller in the vessels with 5·5–11 μg. Mn^{++} and was not visible with 22–33 μg. Mn^{++}. No significant decomposition of H_2O_2 was observed in control experiments. These results are in agreement with the conclusion previously reached that manganese reduces an oxidation product of *p*-cresol and is itself oxidized. In the absence of sufficient Mn^{++} the oxidation product accumulates. In orthophosphate at pH 7 the oxidized manganese reacts stoicheiometrically with H_2O_2. In presence of sufficient Mn^{++}, therefore, accumulation of the oxidation product does not occur and the H_2O_2 is completely decomposed.

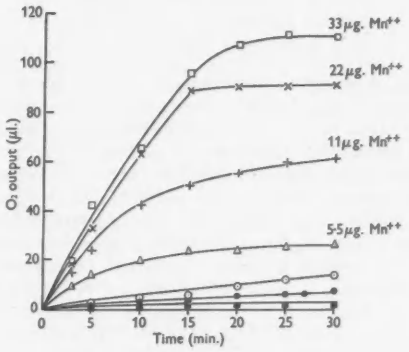


Fig. 5. The decomposition of H_2O_2 by the system peroxidase, *p*-cresol and Mn^{++}. △—△, +—+, ×—×, ⊡—⊡, peroxidase + *p*-cresol with varying Mn^{++} additions; ⊙—⊙, peroxidase heated 10 min. at 100° + *p*-cresol + 22 μg. Mn^{++}; ●—●, peroxidase + 22 μg. Mn^{++} or *p*-cresol + 22 μg. Mn^{++}; ■—■, peroxidase + *p*-cresol; buffer, orthophosphate.

DISCUSSION

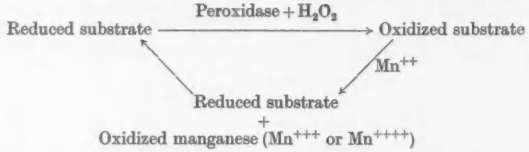
The results obtained provide conclusive evidence that a system is present in horse-radish extract which in the presence of H_2O_2 oxidizes manganese. Colour tests with other root extracts suggest that this reaction is a general phenomenon. The valency of the manganese oxidation product has not been established, but under conditions where manganic manganese is stable the product accumulates in this form. This may arise by a reaction between MnO and MnO_2.

In addition to H_2O_2 a thermolabile and a thermostable factor, which are probably peroxidase and a peroxidase substrate respectively, are necessary for the oxidation. The thermolabile properties of the natural system agree with those attributed to peroxidase by Gallagher (1924) and Herrlinger & Kiermeier (1944), in that prolonged heating at 100° is necessary for complete inactivation. Also, of the root extracts tested those such as horse-radish and turnip, which are good sources of peroxidase, appear to be the most active and the maximum activity as shown by the decomposition of H_2O_2 is reached at low H_2O_2 concentrations.

On the basis of the results obtained with the partially purified peroxidase preparations the working hypothesis is advanced that manganese oxidation is brought about by the oxidized substrate

of the peroxidase, which is involved in a cycle of oxidation and reduction.

The results of the present work suggest a connexion between manganese and plant peroxidase.



It is necessary to postulate such a cycle since only catalytic amounts of substrate are required experimentally. Preliminary experiments suggest that not all peroxidase substrates are active in the cycle. Thus manganese oxidation has been obtained with the system using *o*- and *p*-cresol but not with pyrogallol. The experiments with *p*-cresol indicate that it is an intermediate and not the final oxidation product which brings about the oxidation of manganese.

Comparison of the activities of the horse-radish extracts, used with that of the peroxidase preparation in presence of *p*-cresol, suggests that the activity of the extracts is limited by the amount of the peroxidase substrate present. In agreement with this, activation of comparatively inactive horse-radish extracts could be brought about by the addition of an ether-soluble factor from active extracts.

At the concentrations of H_2O_2 used experimentally part or all of the manganese oxidation product is reduced by the H_2O_2. This involves the Mn^{++} as well as the peroxidase substrate in a cycle of oxidation and reduction. In manometric experiments with small amounts of added Mn^{++} this results in decomposition of the H_2O_2 with evolution of O_2. These experiments were carried out at concentrations of H_2O_2 relatively high compared with those that would be present *in vivo*. At very low concentrations of H_2O_2 it appears probable that the oxidized Mn would react preferentially with metabolites other than H_2O_2. This would lead to a transfer of oxygen from H_2O_2 to plant metabolites without evolution of O_2. Such a transfer would involve the manganese in a cycle of oxidation and reduction and the hypothesis is put forward that this cycle may be responsible, at least in part, for the effect of manganese on plant respiration shown by Lundegårdh (1939). At very low concentrations of H_2O_2, if oxidation is rapid compared with reduction, the oxidation product would accumulate. It is possible that such conditions exist in soils where microbiological oxidation of Mn^{++} leads to an accumulation of manganese higher oxides. It has been suggested that the underlying factor of Mn-Fe antagonism in the growth of plants is the oxidation of ferrous iron to ferric by manganese (Hopkins 1930; Shive 1941). Such a mechanism depends on the presence in the plant of a system oxidizing manganese.

If manganese can also be oxidized through the agency of the peroxidase-like activity of haem or haematin derivatives, or by milk peroxidase, it is possible that manganese oxidation may occur in animal tissues.

It is known that H_2O_2 is formed in several oxidation reactions catalysed by enzymes, e.g. xanthine oxidase, amino-acid oxidase and glucose oxidase. Such H_2O_2 can be used in promoting secondary or coupled oxidations; thus Thurlow (1925) and Harrison & Thurlow (1926) used an enzyme system to provide H_2O_2 for the peroxidase system and Keilin & Hartree (1936, 1945) similarly demonstrated oxidation of alcohols to aldehydes by means of catalase together with H_2O_2 formed by the previously mentioned enzyme systems.

In preliminary experiments colorimetric evidence has been obtained that H_2O_2 formed by the xanthine oxidase—hypoxanthine system may be used to bring about the oxidation of manganese by the horse-radish extract. It seems possible that an enzyme system producing H_2O_2 provides a suitable means of maintaining a low concentration of H_2O_2 and would facilitate the study of the role of the oxidized Mn in plant respiration. Further work along these lines is in progress.

Lastly, it should be pointed out that the system may be able to oxidize metallic ions other than Mn^{++}. It has been possible to follow the oxidation of manganese manometrically, as the oxidation product decomposes H_2O_2, with evolution of O_2. The fact that the other metallic ions so far tested have not shown such activity is no proof that they are not oxidized by the system.

SUMMARY

1. A system which oxidizes manganese in the presence of hydrogen peroxide has been demonstrated in horse-radish root extracts. This system also exists in other root extracts.

2. Under suitable conditions (i.e. in pyrophosphate or citrate at pH 7) the oxidation product accumulates as a coloured manganic complex. Using horse-radish root extract, manganese dioxide was isolated by the dismutation of manganipyrophosphate at weakly alkaline reaction. The oxidation product decomposes hydrazine and can be estimated manometrically by means of this reaction.

3. An increase in the rate of decomposition of hydrogen peroxide was observed on adding small amounts of manganous sulphate to horse-radish extracts. This effect was attributed to manganese oxidation.

4. The manganese oxidizing system in horse-radish extract consists of a thermolabile and a thermostable factor together with hydrogen peroxide. Partially purified peroxidase preparations, in the presence of certain peroxidase substrates and hydrogen peroxide, oxidize Mn^{++}. It is suggested that the thermolabile and thermostable factors in the extract are peroxidase and peroxidase substrate respectively.

5. The hypothesis is advanced that the manganese reduces the oxidized peroxidase substrate and thereby is itself oxidized. This involves the substrate in a cycle of oxidation and reduction. At low hydrogen peroxide concentrations, the manganese oxidation product may react with plant metabolites other than hydrogen peroxide; this would involve the manganese in an oxidation-reduction cycle, which may explain its effect on plant respiration.

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The Biochemistry of Locusts

1. THE CAROTENOIDS OF THE INTEGUMENT OF TWO LOCUST SPECIES (*LOCUSTA MIGRATORIA MIGRATORIOIDES* R. & F. AND *SCHISTOCERCA GREGARIA* FORSK.)

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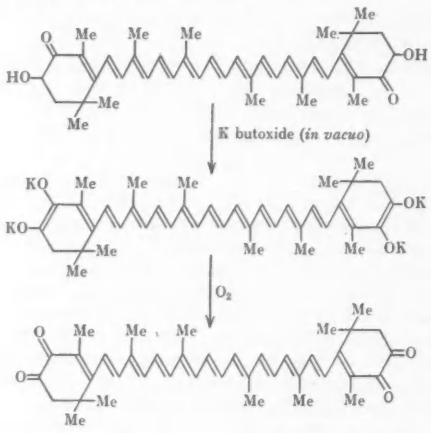
A research programme on the biochemistry of locust pigmentation, with special reference to the problem of swarming, has recently been initiated. An essential preliminary to the study of carotenoid metabolism in these insects (Goodwin, 1949) was the identification of the pigments concerned. Lederer (1935), quoting unpublished work carried out by Volkonsky and himself, reported the presence of a mixture of xanthophylls and carotene in adult *Schistocerca gregaria* Forsk.; Chauvin (1941) noted an unidentified pink pigment in hoppers and a mixture of α- and β-carotenes in adult insects of the same species. No data on *Locusta* species have been recorded.

We have found that hoppers and adult insects of both *Locusta migratoria migratorioides* R. & F. and *Schistocerca gregaria* yielded, on extraction with acetone after removal of the alimentary tract, a mixture of two carotenoids. These pigments have been identified as β-carotene and astaxanthin. Small amounts of lutein (xanthophyll) and chlorophyll were occasionally encountered, but they probably originated in small amounts of unremoved alimentary tract or in small pieces of grass which can become tightly lodged in the buccal cavity and the claws of the forelegs.

The pigments were identified by their chromatographic and spectrographic properties; insufficient

material was available to make crystallization feasible. The most rigid chromatographic criterion applied was that of the mixed chromatogram. When the suspected β-carotene or astaxanthin fraction was admixed with an authentic sample of the corresponding pigment (crystalline β-carotene or astaxanthin isolated from hypodermis of lobster (*Homarus vulgaris* H. M. Edwards) and prawn (*Nephrops norvegicus* L.), and the mixture chromatographed on an appropriate adsorbent, no separation was obtained. The absorption spectra of the purified fractions were compared with those of the authentic pigments over the range 550–400 mμ. in a variety of solvents; in all cases the spectra were almost exactly superposable. In investigations in the carotenoid series, in which the absorption maximum of different components can differ only slightly, it should be emphasized that it is only when the complete absorption curves obtained in different solvents are compared that identification becomes unequivocal; mere recording of maxima is often insufficient.

Astaxanthin was further characterized by its reaction with potassium butoxide *in vacuo* to form a bluish purple enolic salt which on the admission of air is almost instantaneously converted into the orange-coloured astacin (3:4:3′:4′-tetraketo-β-carotene; Kuhn & Sörensen, 1938). Further, treatment of the locust astaxanthin with alkali yielded a product which was chromatographically and spectroscopically identical with astacin produced in a similar way from lobster astaxanthin (Kuhn & Lederer, 1933). Almost all the astaxanthin isolated from locusts was unesterified.



A large number of eyes and wings of *Locusta* and *Schistocerca* were examined separately for carotenoids. All were found to contain β-carotene and free (unesterified) astaxanthin. The astaxanthin in the wings occurs in combination with a protein, but in all probability β-carotene does not.

Examination of the fatty tissues, blood and eggs of *Schistocerca* and *Locusta*, revealed the presence of β-carotene only; neither α-carotene nor any xanthophylls could be detected.

Finally, advantage was taken of the availability of a large number of live locusts to determine whether or not they produce vitamin A. Using the most rigid tests for detecting vitamin A, it was never possible to demonstrate the presence of the vitamin in either species.

EXPERIMENTAL

Extraction, separation and identification of the carotenoids of Locusta *and* Schistocerca

The locusts were killed by cutting off the head just behind the pronotum; the thorax and abdomen were then opened ventrally and the fatty tissues and internal organs removed and discarded. The remaining tissues were thoroughly ground up with acid-washed silver sand and a small amount of anhydrous Na_2SO_4 and extracted 3–4 times with acetone. The combined acetone extracts were concentrated at the pump at a temperature not exceeding 35°; the final traces of acetone were removed at a slightly higher temperature in a stream of N_2. The residue was then dissolved in light petroleum (b.p. 40–60°) and chromatographed using defatted bone meal as adsorbent (Mann, 1943; Goodwin & Morton, 1946; Glover, Goodwin & Morton, 1947). Elution with light petroleum removed the hydrocarbon carotenoids (fraction *A*) and the xanthophylls were subsequently removed from the column with acetone (fraction *B*).

Fraction A was found to have an absorption spectrum identical with that of β-carotene. Its identity with this pigment was confirmed by admixing a portion with a solution containing an approximately equal amount of crystalline β-carotene and chromatographing on activated alumina (Grade O, Peter Spence and Co.); even on slow development down a long column (20–30 cm.) no separation into two fractions could be obtained.

Fraction B was examined in one of three ways:

(*a*) The fraction was dissolved in light petroleum and partitioned between this solvent and 90% (v/v) aqueous methanol The epiphase contained very small amounts of a yellow pigment whilst the main fraction was hypophasic and coloured orange red.

The epiphasic layer was examined spectroscopically and chromatographically; from this examination the presence of small amounts of lutein (xanthophyll) esters was indicated. The identification was not pursued further.

The orange-red methanol solution was diluted with 3 vol. of water and the pigment extracted with light petroleum. The extract, when washed free from acetone with water and dried over anhydrous Na_2SO_4, was chromatographed either on a column of deactivated alumina or $CaCO_3$. (Deactivated alumina was prepared by mixing activated alumina (Peter Spence and Co.) with 10% of its weight of methanol, allowing to stand for 1 hr., filtering the methanol off and drying at room temperature.) Using either adsorbent the pigment was adsorbed as a red zone at the top of the column; elution with ethanol removed a very small amount of brown material which was probably esterified astaxanthin. The

main pigment was eluted with a 2% (v/v) solution of glacial acetic acid in ethanol; this is a characteristic property of astaxanthin (Kuhn & Sörensen, 1938). A very small amount of pink material, which remained on the column, could be eluted with ethanol containing 2·5% (w/v) of KOH; this, according to Kuhn & Sörensen, is a characteristic property of astacin, and its presence in our extracts was probably caused by oxidation of astaxanthin during the working-up process, for astacin does not occur in nature (Kuhn, Stene & Sörensen, 1939).

The glacial acetic acid solution of astaxanthin was diluted with an equal volume of water, and the pigment extracted with ether; this extract was then identified as described below.

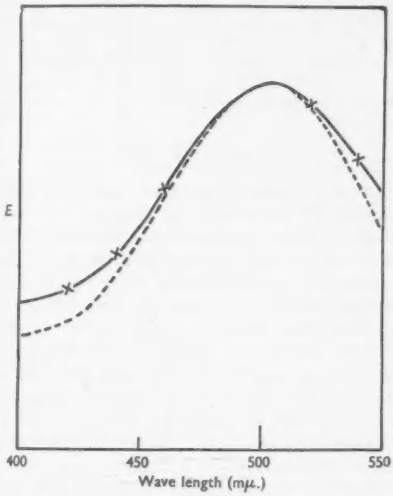


Fig. 1. The absorption spectra of astaxanthin obtained from locusts (-----) and from lobsters (-×-×-×-); solvent, CS_2. Scales for the extinctions (E) have been chosen so that the ordinates representing $E_{max.}$ for the two samples are equal.

(*b*) The extract was dissolved in light petroleum and chromatographed directly, using deactivated alumina as adsorbent. Small amounts of lutein (xanthophyll) were eluted with light petroleum containing 4% (v/v) of acetone; the astaxanthin was then separated and eluted as described under (*a*) above.

(*c*) The light petroleum solution was extracted with a 1% (w/v) solution of Na_2CO_3. This removed astaxanthin from the petroleum phase and at the same time converted it into astacin (Kuhn & Sörensen, 1938). The Na_2CO_3 solution was acidified with acetic acid and the astacin extracted with ether. After removing the ether, the residue was dissolved in light petroleum containing a small amount of ether and chromatographed on deactivated alumina. The pigment was purified by eluting with ethanol containing 2·5% (w/v) of KOH.

The pigment isolated by procedures (*a*) and (*b*) was considered to be astaxanthin and that by procedure (*c*) astacin produced from astaxanthin. Further proof of their identity was obtained: (i) by comparing their absorption spectra with those of authentic samples of astaxanthin and astacin obtained from marine Crustacea (Figs. 1 and 2); (ii) by failure to separate chromatographically a mixture of 'locust' and 'lobster' astaxanthin or astacin into two components; and (iii) by the conversion of astaxanthin into a purple potassium (enol) salt which was rapidly oxidized to astacin in the presence of O_2. To prepare the purple K salt a solution of the pigment in light petroleum was placed in a filter flask closed with a rubber bung from which was suspended by thread a small test tube containing K butoxide. The flask was evacuated to about 1 mm. Hg; the light petroleum was evaporated and the pigment remained as a thin smear on the

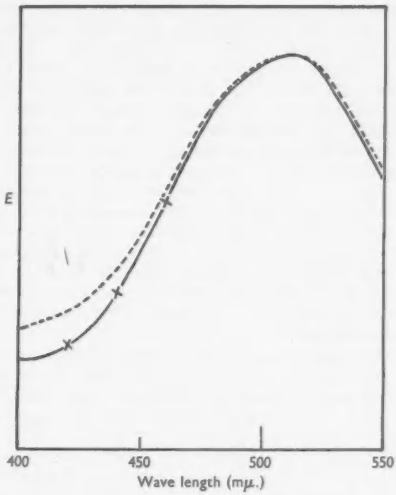


Fig. 2. The absorption spectra of astacin obtained from locusts (-----) and from lobsters (-×-×-×-); solvent, CS_2. Scales for the extinctions (E) have been chosen so that the ordinates representing $E_{max.}$ for the two samples are equal.

bottom of the flask. The flask was then tilted so that the K butoxide poured on to the pigment; immediately a deep purple coloration was produced. On admitting air this immediately turned orange; this orange pigment possessed all the chromatographic and spectroscopic properties of astacin prepared from lobster astaxanthin.

When free and esterified astaxanthin are partitioned between light petroleum and 90% (v/v) aqueous methanol the free form accumulates in the methanol layer (hypophase) and the esterified pigment in the light petroleum layer (epiphase; Kuhn, Lederer & Deutsch, 1933). All the astaxanthin fractions isolated in this investigation were almost completely hypophasic in this test, indicating that the free pigment predominated.

When locust eyes were examined they were treated in the same way as the integuments; the wings, however, were subjected to different treatment.

Examination of locust wings. Various samples of wings from *Locusta*, *Schistocerca gregaria* and one sample of wings

of red locust (*Nomadacris septemfasciata* Serville) were examined in the following way. The wings were placed in a Waring mixer and extracted for 10 min. with light petroleum (b.p. 40–60°); the light petroleum was filtered at the pump through a G 4 sintered-glass filter and examined chromatographically and spectroscopically. The fraction contained only one pigment, β-carotene. The wing residue was divided into two portions, one of which was extracted with acetone in the Waring mixer. The acetone fraction was dark orange in colour and when tested was shown to contain only unesterified astaxanthin. This was good presumptive evidence that the astaxanthin was held attached to a protein.

Consequently, the second portion of the wings was extracted with about 150 ml. water containing two drops of NH_3 solution (sp.gr. 0·88) in the Waring mixer. The resulting mush was centrifuged and the dark brown supernatant liquid decanted off. Addition of solid $(NH_4)_2SO_4$ to saturation threw down a brown protein, leaving the supernatant liquid a pale yellow colour. The protein was centrifuged off and redispersed in a small volume of water. Shaking this solution with light petroleum extracted a small amount of β-carotene. After the protein pigment complex was broken down by the addition of an equal volume of ethanol the pigment was easily extractable with light petroleum. It showed all the properties of free astaxanthin. The wing tissues remaining after extraction with water were extracted with acetone; a small amount of astaxanthin was obtained, probably owing to the presence of unextracted protein. It is important to note that in similar tests with locust integuments it has not yet been possible to detect such an astaxanthin complex.

Body fat and eggs. Samples of body fat and eggs were ground up with anhydrous Na_2SO_4 and the pigment extracted with acetone. On transferring the pigment to light petroleum and chromatographing on activated alumina only one pigment was detected. Spectroscopic data and a 'mixed' chromatogram indicated that the pigment was β-carotene.

Extraction of astaxanthin from lobsters. It was necessary to obtain authentic specimens of astaxanthin with which to compare the locust pigment; lobsters were considered the best source. The methods of extraction are described in a subsequent communication (Goodwin & Srisukh, 1949).

Blood. About 1 ml. of blood was obtained from about fifty immature adult *Locusta*. It was examined according to the method of Glover *et al.* (1947). The only pigment obtainable was β-carotene which was identified in the usual way. The concentration of β-carotene in the blood was approx. 600 μg./100 ml. A similar result was obtained with *Schistocerca* blood, but the β-carotene content was considerably higher, approx. 3000 μg./100 ml.

Tests for the presence of vitamin A in Locusta *and* Schistocerca

A number of acetone extracts of whole *Locusta* and *Schistocerca* were prepared as previously described. After removal of the acetone the extracts were dissolved in light petroleum and chromatographed on defatted bone meal according to the method of Glover *et al.* (1947). Two fractions were obtained, one of which was eluted with light petroleum and which should have contained any esterified vitamin A, and one which was eluted with acetone and should have contained any free vitamin A. The solvents were removed from the two fractions and the residues made up in *cyclo*hexane and examined in two ways: (*a*) the absorption spectrum of the colour produced by reaction of the extract with a $CHCl_3$ solution of $SbCl_3$ was examined in the Hilger-Nutting visual spectrophotometer to determine the wave length of the absorption band, and in the Beckman quartz photoelectric spectrophotometer for the measurement of the extinction at 617 mμ. (i.e. at the $\lambda_{max.}$ for the $SbCl_3$-vitamin A colour test). In no case was an absorption band noted at 617 mμ. nor was any absorption of a transitory nature observed at 617 mμ.; in fact, it always increased considerably on standing. Thus, using the $SbCl_3$ test, no positive evidence of the presence of vitamin A, either in the free or esterified form, could be obtained.

(*b*) The direct absorption spectra of the *cyclo*hexane solutions were recorded using the Beckman instrument. Considerable absorption existed at 328 mμ., but when corrected for absorption not due to vitamin A, most of the curves were 'uncorrectable'; that is, in most cases the absorption calculated as due to vitamin A possessed a small negative value. Thus again no evidence for the presence of vitamin A could be obtained.

DISCUSSION

The present investigation has demonstrated the presence of β-carotene and astaxanthin in two species of locusts, *Locusta migratoria migratorioides* R. & F. and *Schistocerca gregaria* Forsk.; β-carotene exists in the integument, wings, fatty tissues, eggs and eyes, and astaxanthin in the integument, wings and eyes.

Almost all the astaxanthin occurring in the integument is unesterified; it has not yet been possible to demonstrate, as in the case of the wings and lobster cuticle (Kuhn & Lederer, 1933), the presence of astaxanthin as a protein complex in the locust integument.

It is hoped to present in a future paper data concerning the quantitative distribution of β-carotene and astaxanthin in locusts during the various stages of development. As this survey will not include separate data on eyes and wings it is interesting to note here that the mean concentrations in the eyes and wings are, respectively, 13·9 and 5·1 μg./g. of β-carotene and 7·45 and 10·4 μg./g. of astaxanthin. Both the flying wings and the elytra have the same concentration of astaxanthin. Whilst the relative amounts of the two pigments in the eyes are very similar to those found in the integument of adults (Goodwin, 1949) astaxanthin tends to preponderate in the wings. From the figures just quoted it can be calculated that one pair of locust eyes contain 0·130 μg. of β-carotene and 0·048 μg. of astaxanthin; the corresponding values for one set of wings are 0·0255 and 0·356 μg.

The wing astaxanthin occurs in combination with a protein, but in all probability β-carotene does not, for when the ground tissue is extracted with light petroleum (b.p. 40–60°), a solvent which does not liberate carotenoids from their protein complexes, β-carotene, but not astaxanthin, is extracted. When

the residue is extracted in the water containing about 0·05% of ammonia (sp.gr. 0·88) a brown solution is obtained from which a brown protein is precipitated at full saturation with ammonium sulphate. When the brown protein is dissolved in water and denatured by the addition of ethanol, astaxanthin, but not β-carotene, appears in a light petroleum extract. The attachment of astaxanthin to a protein of the wings of the red locust, *Nomadacris septemfasciata* Serville, has also been demonstrated. All these results confirm and extend the claims of Junge (1941) and Okay (1947) that a component of the colour complex of insects, in particular the wings, is an (unspecified) caroteno-protein; that Okay noted such a pigment in the acridid, *Acrida turrita* L., is of special interest. More recently, and following our preliminary notice of the presence of astaxanthin in locusts (Goodwin & Srisukh, 1948), Okay (1948) has stated that the carotenoid of the protein complex in a number of insects is astaxanthin.

The demonstration of the presence of β-carotene only in the fatty tissues and eggs of *Locusta* and *Schistocerca* confirms in general the previous statement of Chauvin (1941) that β-carotene exists in the eggs of *Schistocerca*; however, he also claimed to have detected small amounts of α-carotene. It is interesting to note that investigations on the body fat and gonads of another acridid, the grasshopper (*Melanoplus bivattus* Say), have produced similar results (Grayson, 1942; Grayson & Tauber, 1942).

The carotenoids in locust blood are interesting in two respects. (i) The carotene levels are extremely high compared with those usually obtained for mammals whose blood contains carotene; however, in the only other investigation of the carotene levels in insect blood, Palmer & Knight (1924) found the astonishingly high value of 13,600 μg./100 ml. in the lymph of the potato beetle, *Leptinotarsa decemlineata* Say. (ii) As the blood is bright green and carotene is the only pigment present it follows that it is highly likely that the carotene occurs as a protein complex.

Using the most rigid tests for detecting vitamin A, the antimony trichloride test and the correction procedure of Morton & Stubbs (1946), both carried out using a photoelectric spectrophotometer, it was never possible to demonstrate the presence of vitamin A in either species examined.

Brodskis (1944) and Brodskis & Rungs (1944) recently reported 'traces' of vitamin A in *Schistocerca*; it is suggested that, owing to the somewhat inadequate apparatus at their disposal, these investigators have been unable to distinguish between the colour produced by carotenoids with antimony trichloride and that produced by vitamin A.

The absence of vitamin A from locusts is not unexpected, for up to now the vitamin has never been unequivocally detected and is absent from *Taeniopoda auricornis* Walker (Giral, Giral & Giral, 1943); further, a number of insects can develop normally on diets from which vitamin A or carotene or both are completely absent (McCay, 1938; Bowers & McCay, 1940). The absence from locusts of vitamin A, coupled with the presence of astaxanthin, which is present in their eyes, strongly suggests that the pigment is the photoreceptor in locusts, for Wald (1943) has pointed out that astaxanthin is an almost universal photoreceptor in organisms which do not manufacture vitamin A.

SUMMARY

1. The carotenoids of the integument of the migratory locust (*Locusta migratoria migratorioides* R. & F.) and desert locust (*Schistocerca gregaria* Forsk.) are β-carotene and astaxanthin; the latter occurs in the unesterified form.

2. Both pigments also occur in the eyes and the wings. Astaxanthin exists in the wings as a protein complex.

3. The presence of an astaxanthin protein complex has also been demonstrated in the wings of the red locust *Nomadacris septemfasciata* Serville.

4. Only β-carotene occurs in the fatty tissues, blood and eggs of both species. In the blood it is probably attached to a protein.

5. Preformed vitamin A does not occur in either *Locusta* or *Schistocerca*.

Thanks are due to the Anti-Locust Research Centre for supplies of locusts and a grant towards expenses; to Drs B. P. Uvarov and D. L. Gunn of the Anti-Locust Research Centre for advice and help willingly given; to Mr J. R. Bruce (Marine Biological Station, Port Erin, I.o.M.) for marine samples; and to Prof. R. A. Morton for constant encouragement and advice.

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Some Observations on Astaxanthin Distribution in Marine Crustacea

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During an investigation into the carotenoids of two locust species (Goodwin & Srisukh, 1949) it became necessary to obtain an authentic specimen of astaxanthin for comparative purposes; such a sample was obtained from the lobster (*Homarus vulgaris* H. M. Edwards). As the isolation of this material proceeded it became apparent that the experience of Kuhn & Lederer (1933) on the distribution of free and esterified astaxanthin was not being reproduced; consequently the distribution was reinvestigated in some detail. A similar investigation was carried out on the prawn (*Nephrops norvegicus* L.). At the time the lobsters and prawns were being examined an opportunity arose to examine the pigment of the small red copepod, *Tigriopus fulvus* Fisch. (Fraser, 1936).

EXPERIMENTAL

The first sample of astaxanthin used was obtained from old dry carapaces of lobster with which was mixed a small amount of carapaces of crab (*Cancer pagarus*). The carapaces were softened by placing in approx. 0·2 N-HCl for 24 hr. to dissolve the $CaCO_3$, filtered off, washed with water until free from acid, ground up in a mortar with acid-washed silver sand and anhydrous Na_2SO_4 and extracted 3 times with acetone at a temperature below 30°. The combined acetone extracts were concentrated at the pump, diluted with 2 vol. of water and extracted with light petroleum. When a small sample of the light petroleum extract was shaken with 90% (v/v) aqueous methanol the pigment was almost completely hypophasic; this indicated that the pigment was unesterified, for esterified astaxanthin is epiphasic in this test (Kuhn, Lederer & Deutsch, 1933). A small yellow residue in the epiphase showed no selective absorption in the region 400–450 mμ.

The light petroleum extract was then chromatographed using $CaCO_3$ (A.R.) as adsorbent. Development with light petroleum containing 4% (v/v) of ethanol resulted in the separation of three bands: (1) orange red—most strongly adsorbed, (2) reddish pink, and (3) yellow. The yellow fraction, which was only small, was discarded; it was the same pigment as that found in the epiphase in the partition test. Absorption spectrum measurements in four solvents indicated that the orange-red pigment was astacin and the reddish pink pigment astaxanthin. Although the astaxanthin thus obtained was pure and was identical with the locust pigment, it was considered advisable to compare locust astaxanthin with astaxanthin prepared from fresh material. It was also important to examine fresh material because all our tests indicated that in the carapaces astaxanthin was present in the free form, although Kuhn & Lederer (1933) had claimed that the carapace pigment was esterified. In this experiment the presence of astacin in considerable amounts indicated that oxidative changes must have taken place in the stored carapaces and there was always the possibility that hydrolysis of the original ester (if ester were present) had also occurred.

Three fresh lobsters were killed by immersion in fresh water and the hypodermis removed carefully and as completely as possible from the carapace; the eggs of the two females and the hepatopancreases were also removed for examination.

The hypodermis on extraction with acetone in the manner just described yielded a pigment which was chromatographically homogeneous and epiphasic in a methanol-light petroleum partition. It was the astaxanthin ester previously described by Kuhn and his co-workers; its spectral characteristics were recorded using a number of solvents.

The carapace was divided into two portions. One portion was extracted with acetone after it had first been softened by standing overnight in 2 N-HCl. The pigment extracted was not homogeneous; it consisted of two components, one epiphasic and the other, the major component, hypophasic. Neither component could be further fractionated by chromatography on weakened alumina (Goodwin & Srisukh, 1949). This experiment strongly indicated that the hypophasic fraction was obtained from the carapace itself and that the presence of a small amount of epiphasic pigment was due to contamination with small amounts of hypodermis. In order to confirm this, the second portion of carapace was allowed to stand for 48 hr. in 0·02 N-HCl. This treatment softened the carapace and extracted a small proportion of the astaxanthin-protein complex but not the free pigment. The resulting clear blue solution was filtered off and some

colourless interfering protein removed by addition of $(NH_4)_2SO_4$ to half saturation. The blue astaxanthin-protein complex was then broken down by the addition of ethanol (equal vol.); the solution immediately turned orange and the liberated pigment which remained dispersed in the solution was extracted with light petroleum. On shaking this petroleum solution with 90% (v/v) aqueous methanol the pigment was completely extracted by the methanol. This experiment clearly confirms that the carapace astaxanthin is unesterified.

Lobster eggs were ground up and allowed to stand for some days in contact with water. A green chromoprotein was extracted which exhibited an absorption spectrum with maxima at 476 and 660 mμ.; the spectrum was very similar in shape to that reported by Stern & Salomon (1938) who, however, recorded slightly different maxima, viz. 470 and 640 mμ. Denaturation of the chromoprotein by addition of acetone yielded unesterified astaxanthin, thus confirming the observations of Kuhn & Lederer (1933).

The hepatopancreases were extracted with acetone in the usual way and yielded only very small amounts of a pigment which was, as far as could be ascertained, β-carotene.

Three fresh prawns (*Nephrops norvegicus* L.) were examined in the same way as lobsters. The results obtained were the same, i.e. the hypodermis contained esterified astaxanthin, whilst the carapace contained free astaxanthin attached to a protein.

The carotenoids from *Tigriopus fulvus* were extracted by acetone after grinding with sharp sand. The acetone was removed *in vacuo* and the residue dissolved in light petroleum (b.p. 40–60°), partitioned, and the two fractions purified by chromatography on weakened alumina. Using the methods described previously in this paper, these two pigments were identified as esterified and free astaxanthin; the latter is the major portion of the mixture.

Quantitative measurements were made in order to determine the amount of astaxanthin contained in male and female *T. fulvus*. Using samples of 100 animals, values obtained for males were 5·74 and 4·66 μg./animal and for females 6·0 and 5·77 μg. Gravid females are conspicuous by their egg sacs; consequently two lots of 100 gravid females were collected, separated from their eggs and the two portions examined separately. It was found that whilst females from which sacs had been removed contained 2·93 and 2·00 μg./animal, the sacs themselves contained astaxanthin to the extent of 2·89 and 1·58 μg./sac. From these limited experiments it appears that male and female *T. fulvus* contain about the same amount of pigment, but that gravid females lose about half their store to their eggs.

RESULTS AND DISCUSSION

Whilst Kuhn & Lederer's (1933) claim that the hypodermis of the lobster and prawn and ova of the lobster contain esterified and free astaxanthin, respectively, was confirmed, it was never possible to detect more than traces of astaxanthin ester in the carapaces of either species; the major pigment was always free astaxanthin. It can only be suggested that previous workers had investigated carapaces to which some hypodermis was still adhering. As the pigment concentration in the hypodermis is very much greater than in the carapace, the pigment from small amounts of the former might well have masked that obtained from large amounts of the latter. It was found in the present investigation that complete removal of the hypodermis from the carapace was virtually impossible, and it is considered that the presence of small amounts of esterified pigment in our carapace extracts was due to traces of hypodermis. In order to eliminate the interference produced by the pigments of adhering hypodermis, air-dried carapaces were ground with water containing traces of hydrochloric acid (about 0·02 N) and allowed to stand. The astaxanthin-protein complex was thus extracted without denaturation and the unconjugated hypodermal pigment was not. The resulting pale blue solution was denatured by the addition of ethanol or acetone; the colour changed to orange and the astaxanthin could be extracted with light petroleum. This pigment was completely hypophasic in the phase test, and no esterified astaxanthin could be detected.

Further, the absorption spectrum of the greenish astaxanthin-protein complex (ovoverdin) which occurs in lobster eggs and which is soluble in water has been examined. The spectrum obtained agreed qualitatively with that previously recorded by Stern & Salomon (1938) in exhibiting a broad shallow band in the red region and a much sharper band in the blue-green region of the spectrum. It is in the position of these bands that the present observations differ slightly from those of Stern & Salomon; they record $\lambda\lambda_{max.}$ at 640 and 470 mμ., whilst we record them at 660 and 476 mμ.

Light absorption data on astaxanthin are much less numerous than are those for astacin; Wald (1943) and Karrer & Würgler (1943) record the spectrum in hexane, and Kuhn & Sörensen (1938) in pyridine. However, it is now apparent that the spectrum recorded by Kuhn & Sörensen is incorrect. In order to extend the data, the absorption maximum of astaxanthin in carbon disulphide, pyridine, glacial acetic acid and light petroleum (b.p. 40–60°) are recorded in Table 1. Similarly, only few data have been produced on the spectra of astaxanthin esters (Wald, 1943; Kuhn & Sörensen, 1938) and these indicate that they are very close to that of the parent

Table 1. *Absorption maxima of free and esterified astaxanthin in various solvents*

Solvent	Absorption maximum (mμ.) Free astaxanthin	Esterified astaxanthin
Hexane	470	467–468
Light petroleum (b.p. 40–60°)	470	—
Pyridine	490–491	488
Carbon disulphide	505–506	503
Glacial acetic acid	483–485	481–482
Acetone	475	—

compound. It has now been found that compared with free astaxanthin the esterified pigment occurring in lobster hypodermis shows an absorption band of exactly similar shape, but with its wavelength maximum shifted 1–2 mμ. to shorter wavelengths (Table 1). All the data were obtained using the Beckman photoelectric spectrophotometer.

SUMMARY

1. It has been confirmed that esterified astaxanthin occurs in the hypodermis of the lobster, *Homarus vulgaris* Edw. and the prawn, *Nephrops norvegicus* L., and that the unesterified pigment occurs in the eggs of the lobster.

2. It has not been possible to confirm the presence of esterified astaxanthin in the carapaces of these species; from the evidence presented it is considered that the pigment is in fact free astaxanthin.

3. The lobster hepatopancreas contains only traces of β-carotene.

4. Free and esterified astaxanthin, the former predominating, have been identified in the sea flea, *Tigriopus fulvus* Fisch. In gravid females about 50 % of the pigment is in their eggs.

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The Intermediary Metabolism of the Mammary Gland

2. RESPIRATION AND ACID PRODUCTION OF MAMMARY TISSUE DURING PREGNANCY, LACTATION AND INVOLUTION IN THE RAT

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In a previous paper (Folley & French, 1949*c*) we have shown that slices of lactating mammary tissue respire fairly actively in a medium containing glucose. In the rat the activity is less than that of nervous tissues and kidney, but is of the same order as that of liver; unlike that of liver, however, the respiration is markedly increased over endogenous values in the presence of glucose. The respiratory quotient (R.Q.) of lactating mammary tissue (in glucose) is well above unity in the mouse, rat, rabbit, and, to a lesser degree, the guinea pig, but below unity in ruminants (goat, cow).

Our results on the rat indicated that Q_{O_2}, and perhaps R.Q., is lower in early than in full lactation. This preliminary finding was in line with that of Kleiber, Smith & Levy (1943), who reported a higher respiration for lactating tissue than for tissue taken from pregnant rats, provided the results were calculated to a dry tissue basis. On a moist tissue basis, however, there was no difference in metabolic rate at the end of pregnancy and at the twenty-first day of lactation, because the dry-matter content of the gland was much higher in pregnancy than in lactation.

The relation between the functional activity of a tissue and its respiratory metabolism is of considerable interest, and the mammary gland readily lends itself to a study of this question. We have previously shown (Folley & French, 1949*c*) that, in the rat, experimental depression of lactation due to restriction of the food intake or to adrenalectomy, lowers Q_{O_2}, decreases the R.Q. to values near unity, and increases the aerobic glycolysis. Another approach to this question is to study the metabolism of mammary tissue at various stages of the lactational cycle, using the term in its widest sense to include late pregnancy and post-lactational involution. The present paper reports results of such a study.

METHODS

Animals. Hooded Norway rats undergoing their first lactations were used. The stock diet, fed *ad lib.*, was as described previously (Cowie & Folley, 1948) save that 10 of the parts of whole wheat were replaced by wheat germ. All

litters were reduced to 8 (if possible 4 of each sex) at parturition. Groups were killed (by dislocation of the spine) on the twentieth day of pregnancy (i.e. 1–2 days before parturition), and on days 1, 8, 15 and 22 of lactation, litters being allowed access to the mothers up to the time of autopsy. Obviously the group killed on day 1 may have included some rats which would have failed to lactate successfully had they been allowed to survive. This could hardly have affected the mean results for this group of 10, however, since, of the rats set aside for this work, only about 10% failed to lactate. In order to allow the effects of weaning to be studied in comparison with those of continued suckling, a group was weaned on the twentieth day and killed 2 days later.

General. At autopsy the 3 abdominal* mammary glands from one side were carefully dissected off and weighed, after which samples were taken for determination of retained (extracellular) milk, by determination of the lactose content on a homogenate (Folley & Greenbaum, 1947), and of total dry-matter content (Folley & Greenbaum, 1948). Tissue slices for the manometric experiments were cut from the other 3 glands as described previously (Folley & French, 1949*c*).

R.Q., Q_{O_2} (μl. O_2/mg. final dry wt./hr.) and $Q^{O_2}_{acid}$ were determined on duplicate slices from each rat by the method of Dickens & Šimer (1931) using Dickens & Greville (1933) flasks. The gas phase was 5% CO_2 and 95% O_2, and the medium the Ringer-bicarbonate of Krebs & Henseleit (1932). In all experiments with substrate, 0·3% glucose was used. Determinations in duplicate without substrate were also performed on slices from about half the rats in each group.

RESULTS

Oxygen uptake. Group mean values for $-Q_{O_2}$ are given in Table 1. At the end of pregnancy $-Q_{O_2}$ (glucose) is low, but has increased considerably by the first day of lactation. Thereafter the values rise steadily to a peak level, reached somewhere between the eighth and fifteenth days, which appears to be maintained sensibly constant until the twenty-second day, the slight drop at this time being not statistically significant. By contrast, glands from rats weaned on the twentieth day and killed on the twenty-second day show a marked fall in respiration rate (see also Fig. 1).

The increase in $-Q_{O_2}$ between the twentieth day of pregnancy and the first day of lactation is hardly affected by absence of substrate, but thereafter as lactation progresses $-Q_{O_2}$ increases but slightly, if at all, and it is doubtful whether there is a decrease at weaning. The values for the eighth, fifteenth and twenty-second days of lactation confirm our previous finding (Folley & French, 1949*c*) that glucose markedly increases the respiration of mammary gland slices. Values for the ratio $\frac{Q_{O_2}\text{ (glucose)}}{Q_{O_2}\text{ (no substrate)}}$, given in Table 1, show that this is a property only of the fully lactating gland.

The interpretation of these results is complicated by the changes in the dry-matter content of the mammary tissue (Table 2). Table 2 shows that the fully lactating gland contains less than half the dry matter of the gland at the end of pregnancy, the values agreeing very well with those of Kleiber *et al.* (1943). The question thus arises whether the increase in $-Q_{O_2}$ represents a real increase in respiration rate or is an artifact due to the disappearance of metabolically inert dry matter, such as fat or protein, contained in colostrum stored in the alveoli. On the other hand, there seems no doubt about the reality of the decrease in respiration following weaning, since the increase in the tissue dry matter at this time is relatively slight.

We have attempted to elucidate this problem by calculating for various stages the total respiration of the six abdominal glands, the weight of which, corrected for retained extracellular milk, should remain more or less constant over late pregnancy and lactation, since glandular growth largely ceases by mid-pregnancy (see Folley & Greenbaum, 1947). This approach seems preferable to an attempt simply to express the respiration rate on a moist tissue basis, since the nature of mammary gland slices (see Folley & French, 1949*c*) precludes accurate determination of their moist weights. The total respiration of the abdominal glands is easily calculated for 8, 15 and 22 days of lactation if Q_{O_2}, the total moist weight of the abdominal glands and their retained extracellular milk content, are known. The calculation involves

Table 1. *Respiratory metabolism of slices of rat mammary gland during pregnancy, lactation and involution*

(Errors are indicated in this and succeeding tables by giving mean ± S.E.M.)

		Glucose (0·3%)			No substrate			$\frac{Q_{O_2}\text{ (glucose)}}{Q_{O_2}\text{ (no substrate)}}$
Stage	Days	No. of rats	$-Q_{O_2}$	R.Q.	No. of rats	$-Q_{O_2}$	R.Q.	
Pregnancy	20	10	1·3±0·1	0·83±0·01	5	1·5±0·05	0·62±0·03	0·87
Lactation	1	10	4·4±0·3	1·00±0·05	5	4·0±0·3	0·73±0·01	1·07
	8	8	7·1±0·6	1·62±0·03	4	4·5±0·5	0·76±0·01	1·58
	15	8	10·3±0·4	1·60±0·06	4	5·2±0·4	0·78±0·02	2·03
	22	8	9·6±0·3	1·53±0·03	4	6·3±0·2	0·74±0·02	1·49
Weaning	2	5	5·5±0·9	0·76±0·03	3	5·0	0·64	1·14

* 'Abdominal' refers to the 2 abdominal and 4 inguinal glands.

Table 2. *Total respiration of abdominal mammary glands of rats during pregnancy and lactation*

Stage	Days	No. of rats	Mean body wt. (g.)	Moist wt. of 3 abdominal mammary glands (including milk) (g.)	Milk content of mammary tissue* (%)	Total dry-matter content of mammary tissue (including milk) (%)	Dry-matter content of 'milk-free' mammary tissue (calc.)† (%)	Total respiration of 6 abdominal mammae‡ (μl. O_2/hr.)
Pregnancy	20	10	290	2·56	12·8±0·7	58·1±1·7	—	3,870§
Lactation	1	10	224	3·35	40·4±3·1	37·2±1·8	—	—
	8	8	248	3·12	37·8±3·0	28·6±0·6	30·4	8,370
	15	8	250	3·75	42·2±2·5	25·7±0·3	25·8	11,500
	22	8	250	3·49	57·6±2·1	26·0±0·3	26·6	7,540
Weaning	2	5	251	5·58	41·5±3·6	31·2±0·6	—	—

* Lactose content of rat milk taken as 3·23% (Folley & Greenbaum, 1947).
† Dry-matter content of rat milk taken as 25·60% (Folley & Greenbaum, 1948).
‡ Calculated on the assumption that all the milk in a slice is leached out during the manometric determination.
§ Calculated on the assumption that no colostrum is leached out from a slice during the manometric determination.

a number of reasonable assumptions, the most important of which are the following: (*a*) that the method of Folley & Greenbaum (1947) for calculating the extracellular milk content of mammary tissue from the lactose content of tissue homogenates is reasonably valid; (*b*) that the variations in the total solids and lactose contents of the milk over the period of lactation in question are negligible; and (*c*) that all extracellular milk is leached out from the slice during the respiration experiment. In calculating the milk content of the tissue at these stages the lactose content of rat milk was taken as 3·23% (Folley & Greenbaum, 1947) and the total solids content as 25·60% (Folley & Greenbaum, 1948).

These assumptions are, however, not valid for the gland in late pregnancy because (*a*) the gland then contains colostrum, the composition of which is unknown for the rat, but which by analogy with what is known for the heifer (Engel & Schlag, 1925) is almost certain to contain a very large amount of solids and little lactose, and (*b*) it seems probable that much of the colostrum will remain in the slice throughout the manometric determination. The total respiration of the abdominal glands for the twentieth day of pregnancy was therefore calculated on the assumption that no colostrum is leached out. This has the advantage of giving a maximum value for the total respiration since if, as is probable, some colostrum is lost during the measurements, the figure given is an overestimate. No such calculation was attempted for the first day of lactation since this is probably a period of transition from colostrum to milk, involving unknown and rapidly changing conditions to which no set of assumptions seemed to apply.

The values for the total respiration given in Table 2 indicate an increase of the order of threefold between the end of pregnancy and the fifteenth day of lactation, the increase being somewhat less at the eighth and twenty-second days.

Respiratory quotient. Group mean values are given in Table 1; they are of course independent of changes

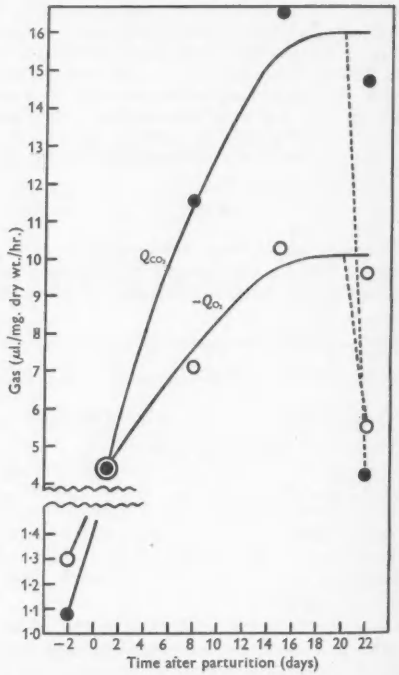

Fig. 1. Gas exchange of rat mammary gland slices during late pregnancy, lactation and after weaning. The points at the ends of the dotted lines refer to groups of rats weaned at the twentieth day of lactation.

in the dry matter or milk content of the tissue. With glucose as substrate the R.Q. is well below unity in late pregnancy, but has risen to unity on the first day

of lactation. By the eighth day the R.Q. has increased to 1·6, a value which is more or less maintained throughout lactation. This value is slightly higher than our previous value for rat mammary gland in full lactation (Folley & French, 1949*c*). After weaning for 2 days the R.Q. has dropped below unity to a value similar to that in late pregnancy. The striking increase in CO_2 output, the increase in Q_{CO_2} above $-Q_{O_2}$ near parturition and the reversal of this change following weaning, which these findings imply, are illustrated in Fig. 1. In the absence of substrate the R.Q. is below unity throughout, although the values for fully lactating tissue are a little greater than those for tissue taken in late pregnancy or after weaning.

Aerobic acid production. Changes in aerobic acid production (Table 3) are mainly considered in terms of ratios of quotients so as to eliminate complications due to changes in the dry-matter and milk content of tissue. We have previously pointed out (Folley & French, 1949*c*) that the acid produced by mammary gland slices probably includes acids other than lactic acid.

Table 3. *Aerobic acid production of rat mammary gland during pregnancy, lactation and involution*

Stage	Days	No. of rats	$Q_G^{O_2}$ (glucose)	$\frac{-Q_{O_2}\text{ (glucose)}}{Q_G^{O_2}\text{ (glucose)}}$	No. of rats	$\frac{-Q_{O_2}\text{ (no substrate)}}{Q_G^{O_2}\text{ (no substrate)}}$	$\frac{Q_G^{O_2}\text{ (glucose)}}{Q_G^{O_2}\text{ (no substrate)}}$
Pregnancy	20	10	0·9±0·1	1·45	5	4·47	2·77
Lactation	1	10	1·4±0·1	3·57	5	9·04	2·90
	8	8	1·8±0·2	4·09	4	9·28	3·76
	15	8	2·4±0·5	5·25	4	12·75	3·08
	22	8	2·0±0·2	5·16	4	6·37	2·40
Weaning	2	5	3·5±0·3	1·55	3	3·51	2·63

The ratio $-Q_{O_2}/Q_G^{O_2}$ (glucose), which gives a measure of the relative changes in respiration and aerobic glycolysis, increases from about 1·5 at the end of pregnancy to a maximum value greater than 5 at the fifteenth day of lactation. Since the total respiration of the abdominal glands increases at least threefold over this period (Table 2), it would seem that the increasing activity of the mammary gland is not accompanied by any appreciable increase in aerobic glycolysis. The extra energy requirements of lactation appear to be met solely by increased oxidation. Table 3 shows that $Q_G^{O_2}$ increases after weaning; since there is also an appreciable increase in the dry-matter content of the tissue at this time (Table 2), the actual increase in glycolysis at weaning is probably somewhat greater than indicated by the quotients.

Similar results were obtained in the absence of substrate, although under these conditions the ratio of respiration to glycolysis is higher. The values for the ratio $\frac{Q_G^{O_2}\text{ (glucose)}}{Q_G^{O_2}\text{ (no substrate)}}$ show relatively little change, but there is an indication of a slight increase in autoglycolysis relative to the glucolysis as the secretory activity of the tissue diminishes.

DISCUSSION

The present results show that, allowing for simultaneous changes in the dry-matter content of the tissue, the onset of lactation in the rat and the subsequent progressive increase in milk yield (see data of Brody & Nisbet, 1938) are correlated with an increase in the metabolic rate of mammary tissue. Our values for Q_{O_2} are at all stages two to three times as great as those of Kleiber *et al.* (1943), possibly because those workers did not use thin slices, so that their tissue may not have been in equilibrium with oxygen. Studies of the oxygen uptake of the ruminant udder by the arteriovenous method show on the whole (e.g. the results of Reineke, Stonecipher & Turner, 1941, on the goat) very little difference between dry and lactating udders as regards arteriovenous differences in oxygen. Our findings on the rat indicate that the enhanced energy consumption in lactation is met by an increase in respiration rather than glycolysis, so in the ruminant, and probably other mammals as well, the increased energy requirements of lactation may involve an increase in blood flow. Jung (1932) reported that the blood flow through the lactating goat udder was about four times the value for the dry gland, an increase in fair agreement with our estimate of a threefold increase in the total respiration of the abdominal glands in the rat.

Even more significant, because in a sense they are qualitative rather than purely quantitative, are the changes in R.Q. (in glucose). The R.Q., well below unity at the end of pregnancy, has risen to unity shortly after parturition and thereafter rises to a value of 1·5–1·6 which is maintained throughout lactation. Despite criticisms of the validity of the R.Q. as an indicator of metabolic processes (Soskin, 1941), it seems safe to conclude that the change in R.Q. of mammary tissue at parturition implies a profound change in the intermediary metabolism of the mammary gland at the start of secretion.

A brief consideration of what this change implies may not be out of place. In previous papers (Folley & French, 1948*a*, 1949*c*) we concluded that the high

in vitro R.Q. of lactating mammary tissue indicates the synthesis of fat from oxygen-rich materials. It is probable, however, that even in late pregnancy, when the R.Q. is low, some fat synthesis is proceeding; and indeed Popják & Beeckmans (1949) has demonstrated the incorporation of ^{14}C into the fatty acids of neutral fat isolated from the mammae of pregnant rabbits given labelled acetate. There thus appears to be a turnover of fat in the gland even during pregnancy, but the high R.Q. following parturition given by slices of ruminant udder in the presence of acetate and of non-ruminant gland in the presence of glucose (Folley & French, 1948*b*, 1949*a*) undoubtedly indicates net synthesis of fat by lactating tissue. We have suggested that part at least of the milk fat, perhaps particularly the shorter-chain fatty acids which distinguish it from body fat, is synthesized in the mammary gland itself from acetate (Folley & French, 1948*b*, 1949*a*) which the ruminant absorbs in large quantities from the rumen. Since Bloch & Rittenberg (1945) have shown in the rat that considerable amounts of acetate are continually produced, it seems likely that acetate is a substrate for synthesis of milk fat by the mammary gland in all mammals. The above-mentioned experiments of Popják & Beeckmans (1949) confirm this for the rabbit. The apparent ability, as indicated by the high R.Q., of mammary slices from non-ruminants, to effect net synthesis of fat from carbohydrate *in vitro* (Folley & French, 1948*a*, 1949*c*) would be explicable if the pathway from carbohydrate to fat passes through acetate. Bloch (1947) does not favour this possibility, but the position is admittedly rather obscure, and further *in vitro* work on the mammary gland might well help in clarification. The main difficulty hitherto has been the inability of lactating tissue from non-ruminants to utilize acetate *in vitro* in contrast to slices from ruminant udders (Folley & French, 1948*b*, 1949*a*); but in recent experiments (Folley & French, 1949*b*) we have shown that lactating mammary slices from rabbit and rat will utilize acetate in presence of small concentrations of glucose, the R.Q. being above unity, and in the rabbit often greater than in the presence of glucose alone. It seems that mammary slices from various species are subject to different limiting conditions, and it may be that non-ruminant mammary tissue, which contains very little glycogen (Folley & French, unpublished), requires glucose to provide the glycerol necessary for glyceride synthesis, a process which may well favour the synthesis of fatty acids. Other possibilities are that glucose is necessary to provide additional carbon for fatty acid synthesis, as suggested by Bloch (1947), or to provide energy for the activation of acetate.

The onset of lactation involves a change from a condition characteristic of late pregnancy in which, although cytologically the secretory phase has begun, the product bears little resemblance in composition to milk, to the post-parturient state in which, provided suckling proceeds, large quantities of normal milk are secreted. These events are accompanied by the following changes in the *in vitro* metabolic properties of the rat mammary gland: (*a*) the metabolic rate increases to an extent difficult to assess from our data, but probably at least threefold; (*b*) the slices acquire the property of responding to glucose in the medium by an increase in respiration; (*c*) the R.Q. increases above unity; and (*d*) the ratio of respiration to glycolysis increases.

The initiation of lactation is under endocrine control, the chief feature being an increased release of prolactin, and perhaps of other hormones concerned in lactation, by the anterior pituitary (see review by Folley, 1947*a*), and it would therefore seem likely that a close relationship must exist between the lactogenic hormone or hormone complex and the changes in the mammary gland metabolism occurring at parturition.

The changes which follow weaning are also of interest in this connexion. Within a short time of removing the litter the mammae exhibit changes in metabolism in the opposite direction from those which occur at parturition; the respiration decreases and, more significant, the R.Q. quickly drops below unity. Weaning also causes a definite increase in the apparent aerobic glycolysis similar to that shown previously (Folley & French, 1949*c*) to be associated with the partial inhibition of lactation resulting from inanition or adrenalectomy. Changes in mammary metabolism following weaning may be due to (*a*) the loss of the suckling stimulus, and (*b*) the effects, chemical or physical, of non-removal of milk. No attempt has been made in the present work to assess the relative role of these two primary factors, but obvious test experiments, utilizing the techniques of Selye (1934), suggest themselves. The suckling stimulus is believed to influence the function of the mammary gland through a neurohormonal arc involving as its final, centrifugal link the release of prolactin by the anterior hypophysis (see Folley, 1947*b*, for review); and the experiments of Selye (1934), and others, suggest that removal of the suckling stimulus may be the more important of the two above-mentioned factors, at any rate in the early stages of weaning. Thus the changes in the R.Q. of mammary tissue at parturition and after weaning alike raise the question how far the physiological action of prolactin on the mammary epithelium, an action which the experiments of Lyons (1942) and Meites & Turner (1948) involving intramammary duct injection of prolactin show to be direct, is bound up with the promotion of reactions leading to synthesis of fat from oxygen-rich materials. Mammary tissue may well prove particularly useful for an attack on this aspect of what is undoubtedly the

outstanding problem of endocrinology, the hormone-enzyme relationship.

The results for the unweaned group at 22 days of lactation seem to indicate that the respiration and R.Q. have begun to decline; but in order to study the normal course of lactation beyond this point it would be necessary to provide fresh litters so as to eliminate the effect of self-weaning of the young. (Incidentally, self-weaning is shown by the increased milk content of the glands at 22 days, Table 2.)

In conclusion, we may refer to the value of a more or less complete picture of changes in the respiratory metabolism of the mammary gland in relation to various phases of its physiological activity, such as is provided by this work, as forming a background for further studies of the biochemical mechanisms involved in the synthesis of milk.

SUMMARY

1. The respiratory metabolism and acid production of slices of rat mammary gland in the presence and absence of glucose have been studied during pregnancy, lactation and following weaning.

2. $-Q_{O_2}$ is low at the end of pregnancy, but has increased considerably on the day following parturition; thereafter the values rise to a maximum value of approximately 10 at mid-lactation. Interpretation of these changes is complicated by changes in the dry-matter content of the tissue, which by mid-lactation has fallen to less than half of the value at the end of pregnancy. However, calculation of the total respiration of the abdominal mammary glands indicates that the initiation and subsequent increase in the intensity of lactation are accompanied by a true increase in the respiration.

3. The effect of glucose in increasing the respiration of mammary gland slices is only seen in lactating tissue.

4. The R.Q. (in glucose) is well below unity at the end of pregnancy, but has risen to unity on the day following parturition; thereafter it rises to a maximum of approximately 1·6, which is maintained throughout most of the lactation period. In absence of substrate, the R.Q. remains below unity throughout lactation.

5. Lactating gland shows a higher value for $Q_{O_2}^{O_2}$ than the gland at the end of pregnancy; the increase is, however, largely an artifact due to the changes in the dry-matter content of the tissue. The extra energy requirements of the lactating gland seem to be met solely by increase in respiration.

6. Weaning is followed by a sharp fall in respiration, the change in Q_{O_2} being too great to be attributed to the relatively slight increase in the dry-matter content of the tissue which occurs at this time. Further, the R.Q. decreases below unity and the apparent aerobic glycolysis rises.

7. The significance of the results is discussed in the light of possible mechanisms of fat synthesis in the mammary gland, and of relationships between anterior-pituitary hormones and changes in mammary gland metabolism.

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Tryptophan and the Biosynthesis of Nicotinamide*

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There has been much discussion as to how tryptophan affects nicotinamide metabolism. It is generally believed that a direct conversion of tryptophan into nicotinamide takes place and many experiments have been carried out to elucidate the route of this conversion. Ellinger & Abdel Kader (1949*c*) observed that in *Escherichia coli* the biosynthesis of nicotinamide from ammonium lactate and ornithine was completely inhibited by DL-2-, 4-, 5- and 7-methyltryptophans in 2 mM-concentrations, growth not being affected. This inhibition suggested, in the light of Fildes's (1940) theory of metabolite antagonism, that tryptophan is actively involved in the biosynthesis of nicotinamide. Heidelberger, Gullberg, Morgan & Lepkowsky (1948) found recently that after administration of DL-tryptophan labelled in the β-position of the side chain with ^{14}C to dogs, rabbits and rats, kynurenine and kynurenic acid eliminated in the urine contained the labelled carbon atom, but that the urinary nicotinamide methochloride was free from radioactivity. These results suggested the possibility of tryptophan not being directly converted into nicotinamide, but having a catalytic coenzyme-like action in the biosynthesis of nicotinamide. The present paper deals with experiments to test this hypothesis.

EXPERIMENTAL

Experiments were carried out *in vitro* with mixed cultures from rat caecum content and with pure cultures of *E. coli*, and *in vivo* with rats.

Material used. The mixed cultures of rat caecum content were prepared in the following way: rats which had been found to have a high nicotinamide methochloride elimination were gassed, and immediately afterwards the content of the caecum was placed in weighing bottles under sterile conditions. One portion (about 200 mg. wet weight) was ground in a mortar with 10 ml. peptone water to form a homogeneous suspension and the rest was dried to constant weight in order to calculate the dry weight of the suspended material. From the suspension and from dilutions prepared from it agar plates were inoculated and smears were made in order to obtain qualitative and quantitative information about the organism present. There was a great variety of organisms contained in the mixed cultures. The most frequent types were hitherto unidentified cocci growing on agar in minute colonies, various strains of streptococci, staphylococci and coliform bacilli. Their relative frequency in the caecum content was about 8000:160:80:1, respectively. In addition, a number of other organisms (acidophilus, *Proteus*, Gram-positive cocci and diplococci, Gram-negative oval cocci and Gram-positive bacilli) were occasionally found. The *E. coli* used were the strains 3*c* and 4*c* isolated from human and rat faeces respectively, which were classified as type I faecal (Ministry of Health, 1939). The bacteria were grown on agar slopes and fresh cultures were made in ammonium lactate for inoculation.

Six rats used in the *in vivo* experiments were cross breds from the hooded Lister stock and P. Ellinger's albino stock, of both sexes, about 4 months old and weighing 280–320 g. They were kept on the mixed diet described previously (Ellinger, Fraenkel & Abdel Kader, 1947). Their average daily nicotinamide methochloride output and their response to nicotinamide had been examined for several months.

Salt solutions. The following solutions were used for incubation: (1) saline solution: 0·89% (w/v) NaCl; (2) ammonium lactate solution (Fildes, 1938); (3) saline phosphate solution: KH_2PO_4, 4·5 g., NaCl, 1·0 g.; $MgSO_4$, 0·02 g.; $FeSO_4$, 0·02 g., in 400 ml. water, adjusted with NaOH to pH 7·6; 4 ml. of this solution were made up to 10 ml. with water; (4) saline phosphate glucose solution: glucose was added to solution (3) to a final concentration of 1 mM.; (5) casein hydrolysate solution (Barton-Wright, 1944).

Analytical methods. Estimations were carried out in duplicate. The growth of bacteria was measured by comparing the opacity of a suspension with that of a standard (Brown, 1919). Nicotinamide was assayed microbiologically (Barton-Wright, 1944) either in the medium alone after removing the cells on the centrifuge, or in the medium and organisms after autoclaving. Nicotinamide methochloride was measured by the acetone method (Huff & Perlzweig, 1947). Free tryptophan (without indole) was estimated by the method of Horn & Jones (1945). DL-Tryptophan from different sources was used: for the rat experiments, commercial samples from Glaxo Laboratories Ltd. and from Roche Products Ltd., unpurified; for bacterial experiments the same samples, unpurified and purified by repeated recrystallization, and one sample prepared and kindly supplied by Dr R. L. M. Synge. No difference was observed in their action.

General arrangement of the experiments

In order to measure the consumption of tryptophan during the synthesis of nicotinamide, casein hydrolysate and ammonium lactate solutions without or with the addition of 2 mM-DL-tryptophan, and 2 mM-DL-ornithine dihydrochloride were inoculated with a heavy inoculum of pure cultures of *E. coli* 4*c*, the saline washings of broken *E. coli* 4*c* or a mixed culture from the rat caecum and incubated at

* Some of the results presented in this paper were communicated to the Biochemical Society on 4 December 1948 (Ellinger & Abdel Kader, 1948).

37° for 48 hr. Nicotinamide and free tryptophan in the solution were measured before and after incubation.

The effect of washing the cells on their ability to synthesize nicotinamide from ammonium lactate and on the effect of tryptophan on this synthesis was studied. A 48 hr. culture of *E. coli* in casein hydrolysate was centrifuged and the organisms were washed with water. Samples of the suspension (75×10^8 organisms/ml.) were examined for nicotinamide production in an ammonium lactate medium in the absence and presence of 2 mM-DL-tryptophan, of the unwashed cells and 3, 6 and 9 times washed cells. The samples were prepared by adding 0·5 ml. of a culture containing 15×10^8 organisms/ml. to 9·5 ml. of salt solution.

The dependence of the nicotinamide formation by washed *E. coli* on the concentration of the tryptophan was examined in the following way: *E. coli* 4*c*, washed 3 times with water, were suspended in ammonium lactate solution containing various concentrations of DL-tryptophan, incubated at 37° for 48 hr. and the nicotinamide content was measured. In order to obtain more detailed information on the biosynthesis of nicotinamide when the enzyme system involved was freed from all possible substrates, fresh *E. coli* cells washed 4 times with water and then suspended in saline were disintegrated mechanically (Curran & Evans, 1942). A portion of the broken cells was washed several times with saline, part of the washings was dialysed through cellophan against saline under sterile conditions at room temperature for 48 hr. The outside solution was changed 3 times and continuously stirred mechanically during the dialysis. The completeness of the disintegration was examined by plating and taking viable counts at the various steps. The washed cells, the broken cells, the saline washings before and after dialysis and the exhaustively washed cell fragments were used for one or other of the following experiments. They were incubated in either saline phosphate, saline glucose phosphate or ammonium lactate solutions in the absence or presence of DL-tryptophan, DL-ornithine dihydrochloride or both at 37° for 48 hr. The nicotinamide content of the solution was estimated after incubation.

Rat experiments. The rats were kept in metabolism cages, the urine was collected daily and nicotinamide methochloride elimination was determined. At intervals of 3 or 4 days the rats were given either DL-tryptophan or nicotinamide in doses rising from 20 to 400 mg. and from 2·5 to 50 mg. respectively. These compounds were administered either orally by mixing them thoroughly with the food and controlling the completeness of the consumption, or intraperitoneally.

RESULTS

Experiments with bacteria

In all microbiological experiments two to three parallel experiments were carried out which gave similar results to those reported in detail. Experiments to relate the consumption of tryptophan to the synthesis of nicotinamide during incubation are given in Table 1. Although the growth of the pure cultures of *E. coli* used was good, no marked diminution of tryptophan was observed with the method used, which is rather insensitive but is probably the best existing procedure. 20 μg./ml. is the lower limit of assay and the standard deviation is about ± 5 %. By concentrating the salt solutions to be examined *in vacuo*, amounts of 1 μg./ml. might be measured with a standard deviation of about ± 10 %. Similar experiments carried out with washed cells and washings of broken cells also showed no detectable consumption of tryptophan during incubation, while measurable amounts of nicotinamide were formed. However, the amounts of nicotinamide formed are very small compared with those of the tryptophan present in the medium and the method used for the former is about ten thousand times more sensitive than that employed for tryptophan. The results with pure *E. coli* are therefore not conclusive. By contrast the tryptophan consumption of mixed cultures (Table 1) during nicotinamide synthesis in both ammonium lactate-ornithine-tryptophan and in casein hydrolysate solution is definite and outside the limit of error of the method. Growth was also considerable in these experiments.

When *E. coli* cells were washed repeatedly with water they gradually produced less nicotinamide (Table 2). The nicotinamide synthesis, however, was always restored to the level of the unwashed cells when 2 mM-tryptophan was added; with the washed cells no growth was observed. The relative effect of tryptophan on the synthesis of nicotinamide as

Table 1. *Content of tryptophan and nicotinamide of salt solutions before and after incubation*

Inoculum	Salt solution	Tryptophan content of salt solution (μg./ml.)		Nicotinamide content of salt solution and organisms (μg./ml.)	
		Before incubation	After incubation	Before incubation	After incubation
E. coli 4*c*	Ammonium lactate	<20	<20	10	32
	Ammonium lactate + ornithine (2 mM) + DL-tryptophan (2·2 mM)	442·0	441·4	8	49
	Casein hydrolysate	87·6	87·7	25	40
Saline washings of broken-up *E. coli* 4*c*	Ammonium lactate + DL-tryptophan (1·9 mM)	382·8	383·1	0	27
Mixed cultures from rat caecum	Ammonium lactate	<20	<20	15	31
	Ammonium lactate + DL-ornithine (2 mM) + DL-tryptophan (2·2 mM)	440·9	385·4	12	33
	Casein hydrolysate	87·7	71·2	10	25

compared with the control (without tryptophan) is greater in washed than in untreated cells. This effect is presumably due to the decreased nicotinamide synthesizing activity of the cells, caused by the washing.

Concentrations of DL-tryptophan up to about 0·1 mM had no stimulating effect on the nicotinamide synthesis by washed *E. coli* in ammonium lactate solution. At a concentration of 0·3 mM a marked increase was observed and the stimulating effect reached its maximum at concentrations of 1–2 mM. Higher concentrations did not produce any further increase in nicotinamide synthesis.

Table 2. *The effect of repeated washings of* E. coli 4c *on the nicotinamide synthesis in ammonium lactate solution and on its increase by tryptophan*

(In all tubes the concentration of the cells was about 40×10^6 organisms/ml.)

	Nicotinamide content of salt solutions and organisms (μg./ml.)			
No. of washings ...	0	3	6	9
Salt solution				
Ammonium lactate	28	15	7·5	5·0
Ammonium lactate +2 mM-DL-tryptophan	28	28	29	28

Table 3. *The effect of various concentrations of* DL-*tryptophan on the nicotinamide synthesis in ammonium lactate solution by washed* E. coli 4c

Tryptophan concentration (mM)	Nicotinamide content of salt solution and organisms (mμg./ml.)
0	10
0·1	11
0·3	15
1·0	25
2·0	25
5·0	24
10·0	24

Table 4. *Nicotinamide synthesis by washed and by broken* E. coli 4c *in ammonium lactate solution in the absence or presence of ornithine, tryptophan or both*

Compound added	Washed *E. coli*; nicotinamide content of salt solution and organisms (mμg./ml.)	Broken *E. coli*; nicotinamide content of salt solution (mμg./ml.)
None	6·3	7·5
DL-Ornithine (2 mM)	50·0	50·0
DL-Tryptophan (1 mM)	32·5	32·5
DL-Tryptophan (2 mM)	38·5	35·5
DL-Ornithine (2 mM) + DL-Tryptophan (1 mM)	49·0	48·0
DL-Ornithine (2 mM) + DL-Tryptophan (2 mM)	49·0	50·6

Synthesis of nicotinamide was also observed on incubation of washed *E. coli*, broken cells (Table 4), and undialysed or dialysed washings (Table 5) of disintegrated cells, in ammonium lactate solution alone or with either tryptophan, ornithine or both tryptophan and ornithine. Ornithine increased the synthesis considerably as did tryptophan, but the synthesis in the presence of ornithine was not markedly affected by simultaneous addition of tryptophan. When the same experiment was carried out in saline phosphate solution no nicotinamide was formed in the absence or presence of tryptophan alone, but nicotinamide was produced in the presence of ornithine by undialysed and dialysed cell washings. No nicotinamide was formed in saline phosphate solution containing tryptophan by washed cells even in the presence of glucose, or by saline washings of broken cells. No nicotinamide was formed by washed cells, broken cells or cell washings in 1% saline solution in the presence of ornithine or tryptophan or both of them. No nicotinamide was formed by the exhaustively washed cell fragments in either solution with or without added tryptophan or ornithine.

The enzyme systems involved in the nicotinamide synthesis were contained in the saline washings of the broken cells and retained their nicotinamide synthesizing activity after dialysis. *E. coli* 3c behaved similarly to *E. coli* 4c.

Experiments with rats

When rats (Table 6) were given rising doses of nicotinamide by mouth the urinary elimination of nicotinamide methochloride increased proportionately to the dose administered. The increase varied individually in the different rats, but in the same rat it rose in proportion to the dose given for doses between 2·5 and 50 mg. The response to intraperitoneally administered nicotinamide was in all six rats lower than when nicotinamide was given by mouth. When tryptophan was given by mouth in doses of 50 mg. and more, there was an increase in nicotinamide methochloride output proportional to the dose administered up to about 200 mg. Above this level no further increase of nicotinamide methochloride elimination occurred in any of the six rats. When tryptophan was injected intraperitoneally no increased nicotinamide methochloride output was observed in either rat after doses up to 100 mg. and only a very slight increase in two rats after doses of 200 mg.

DISCUSSION

Ellinger & Abdel Kader (1949*a*) showed that tryptophan increased the nicotinamide synthesis by mixed cultures from the rat faeces or caecum content, but not that by pure cultures of *E. coli*. This action had been attributed to an ability of some organisms of the mixed cultures, but missing from *E. coli*, to split the tryptophan molecule in such a way that the

Table 5. *Nicotinamide synthesis by undialysed saline washings of broken cells of* E. coli 3c *and* 4c *and dialysed washings of* E. coli 4c *in ammonium lactate and saline phosphate solutions in the absence and presence of* DL-*ornithine and* DL-*tryptophan*

Salt solutions	Compound added	Nicotinamide content of washings: Undialysed *E. coli* 3c (mμg./ml.)	Undialysed *B. coli* 4c (mμg./ml.)	Dialysed *E. coli* 4c (mμg./ml)
Ammonium lactate	None	6·3	10·5	12·5
	DL-Ornithine (2 mM)	35·0	40·0	33·5
	DL-Tryptophan (2 mM)	25·0	37·0	40·0
	DL-Ornithine (2 mM) + DL-tryptophan (2 mM)	35·0	40·0	35·0
Saline phosphate	None	0	0	0
	DL-Ornithine (2 mM)	15·0	25·0	12·5
	DL-Tryptophan (2 mM)	0	0	0
	DL-Ornithine (2 mM) + DL-tryptophan (2 mM)	15·0	26·0	12·5

Table 6. *The urinary elimination by rats of nicotinamide methochloride following oral or parenteral administration of nicotinamide or* DL-*tryptophan*

Compound tested	Dose (mg.)	Route of administration	Increase in nicotinamide methochloride output (mg.): Rat 1	Rat 2	Rat 3	Rat 4
Nicotinamide	2·5	Oral	1·704	1·099	1·068	2·062
	5·0	Oral	5·128	2·423	2·524	5·146
	10·0	Oral	8·138	5·871	6·249	9·402
	20·0	Oral	15·133	10·210	11·947	16·670
	50·0	Oral	28·389	18·280	28·885	40·117
	10·0	Intraperitoneal	2·931	4·136	5·424	5·688
DL-Tryptophan	20	Oral	0	0	0	0
	50	Oral	1·246	0·631	0·300	1·530
	100	Oral	3·132	1·395	0·450	2·400
	200	Oral	5·909	2·565	1·149	4·076
	400	Oral	6·493	2·550	1·125	3·039
	50	Intraperitoneal	0	0	0	0
	100	Intraperitoneal	0	0	0	0
	200	Intraperitoneal	0·840	0·085	0	0

Experiments with two more rats revealed essentially similar results.

latter could be utilized by *E. coli* for the synthesis of nicotinamide. When washed coli, instead of untreated coli, were examined for their ability to synthesize nicotinamide and the action of tryptophan on this synthesis, it was found that tryptophan increased nicotinamide production by the washed cells as compared with the control in ammonium lactate solution in a similar way as was found with mixed cultures. The systematic examination of the effect of repeated washings on the nicotinamide production by *E. coli* and on the tryptophan action on the synthesis (Table 2) showed that washing gradually diminished the synthesizing power of the organism and that this was restored by added tryptophan. The apparent relative increase in nicotinamide synthesis by added tryptophan as compared with that in ammonium lactate alone was, therefore, due to the restoration of tryptophan removed by washing. This fact indicated that the stimulating effect of tryptophan on the nicotinamide synthesis by mixed cultures might possibly be due to a reduced tryptophan concentration of the medium. That this was so, was shown by investigation of the tryptophan content after incubation of mixed cultures (Table 1). This was considerably reduced during incubation and nicotinamide synthesis. Whether the unchanged tryptophan content of the salt solutions of pure *E. coli* cultures during growth and nicotinamide synthesis was due to tryptophan formation equal to tryptophan destruction or to the fact that changes were too small to be detected by the method of assay could not be decided.

The experiments show, at least, that with pure cultures of *E. coli* nicotinamide synthesis is not accompanied by a marked consumption of tryptophan as in the case of mixed cultures. The concentration of tryptophan optimal for maximum nicotinamide formation was found to be 1–2 mM (Table 3) and the concentration of about 2 mM was, therefore, used in all experiments.

The facts discussed so far, namely, that tryptophan stimulates nicotinamide synthesis by *E. coli* in ammonium lactate solution only when its concentration in the cells is suboptimal, that its concentration

in mixed cultures is suboptimal and consequently the nicotinamide synthesis by these mixed cultures is stimulated by added tryptophan and that rising concentrations of added tryptophan increase nicotinamide synthesis by washed coli cells proportionally to its concentration only until the optimal level is reached, can best be explained by a catalytic action of tryptophan in the nicotinamide synthesis by *E. coli*. Moreover, the fact that no nicotinamide is formed by undialysed or dialysed washings of broken cells when tryptophan, but no other source of nitrogen, is present in the salt solution (Table 5) supports this hypothesis.

As had been shown (Ellinger & Abdel Kader, 1949*a*) nicotinamide synthesis by *E. coli* is independent of the intactness of the cell structure. This is confirmed by the results shown in Table 5. Moreover, the fact that the enzyme system is contained in the washings of the broken cells and can be dialysed without loss of activity, made it possible to remove all soluble and dialysable substrates from the enzymes.

It had been suggested that ornithine might be an intermediate in the biosynthesis of nicotinamide (Klein & Linser, 1932; Guggenheim, 1940; Ellinger & Abdel Kader, 1949*a*). In the presence of ornithine, tryptophan had very little or no effect on nicotinamide synthesis by either of the coli preparations. If ornithine is formed as an intermediate the findings would mean that tryptophan is mainly involved in the early stages of the nicotinamide synthesis, i.e. from ammonia and lactate to ornithine.

The experiments on rats (Table 6) confirmed the findings of Schweigert & Pearson (1947) and of Ellinger & Abdel Kader (1949*a*) that tryptophan increases the nicotinamide methochloride output to a far higher degree when given orally than parenterally. The differences were, however, much greater than described before; of six rats only two showed a slight increase after an intraperitoneal administration of 200 mg. DL-tryptophan. These results indicate clearly the importance of the intestinal flora for the nicotinamide-saving action of tryptophan. If the tissues are concerned with this action at all, this can only be of secondary importance.

The fact that rising doses of tryptophan increase the nicotinamide formation, as indicated by the urinary elimination of its methyl derivative, up to a maximum which could not be raised by larger doses of tryptophan though it could be by nicotinamide application, indicated that a kind of tryptophan saturation has to be reached to obtain optimum nicotinamide formation. The maximum was reached in each of six rats of about 300 g. when 200 mg. of DL-tryptophan were orally administered. If one were to assume an even distribution of the ingested tryptophan over the whole body, this would mean that saturation was reached at a concentration of 3·3 mM. This assumption of even distribution is certainly incorrect and a comparison between rat and *E. coli* is not admissible. But it may be noted that in both rat and *E. coli* maximum nicotinamide formation was observed with tryptophan concentrations of the same order. The results with the rat also support the conception of a catalytic action of tryptophan in nicotinamide biosynthesis. However, there are some apparent objections to this conception: a catalytic action would mean that tryptophan would play the role of a coenzyme in the nicotinamide synthesis and the methyltryptophans substituted in the indole nucleus would, therefore, be metabolite antagonists to a coenzyme. No such case has been described so far. The concentration of tryptophan producing optimal nicotinamide synthesis compared with the amount of nicotinamide synthesized is high and this is unusual for a catalyst. But catalytic reactions are known which require a fairly high concentration of the catalyst, e.g. nickel in catalytic hydrogenation or aluminium chloride in Friedel and Craft's reaction.

SUMMARY

1. The effect of tryptophan has been studied on the biosynthesis of nicotinamide by washed and broken *E. coli*, by undialysed and dialysed washings and washed fragments of the broken cells, in various salt solutions.

2. The effect of rising doses of tryptophan on the nicotinamide methochloride elimination by rats has been examined.

3. Nicotinamide was formed by washed and broken *E. coli* and by their undialysed and dialysed washings, but not by exhaustively washed cell fragments, in salt solutions containing sources of nitrogen other than tryptophan, such as ammonium ions or ornithine; the formation was increased by the addition of tryptophan.

4. No nicotinamide was formed by these cell preparations in the presence of tryptophan when no other source of nitrogen was present.

5. The effect of rising doses of tryptophan on the nicotinamide synthesis by washed *E. coli* reached a maximum at concentrations of 1–2 mM.; a further increase of the concentration did not increase the synthesis of nicotinamide.

6. In rats comparative experiments on the effect of tryptophan administered either orally or intraperitoneally confirmed the importance of the intestinal flora for the action of tryptophan on the nicotinamide metabolism. By feeding rising doses of tryptophan it was shown that as in *E. coli* a saturation point could be reached.

7. The bearings of these findings on the possible role of tryptophan in the biosynthesis of nicotin-

amide are discussed. It could best be explained by a kind of catalytic action of tryptophan on the nicotinamide synthesis

NOTE ADDED 7 APRIL 1949

Since this paper was submitted, Heidelberger, Abraham & Lepkowsky (1948) have reported feeding DL-tryptophan labelled in the β-position of the side chain with ^{14}C to rats; nicotinamide methochloride eliminated after ingestion contained a labelled C atom in the carboxylic acid group. This does not, however, prove, as is claimed by these authors, that 'the conversion proceeds in the same fashion in the rat as in Neurospora'.

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Some Observations on the Amino-acid Distribution of Collagen, Elastin and Reticular Tissue from Different Sources

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By histological methods three types of connective tissue can be shown to be present in skins; collagen, elastic fibres and reticular tissue. Collagen, in the form of white bundles of interweaving fibres, makes up the greater part of the skin; the elastic fibres are pale yellow in colour and occur mainly in the grain layer; reticular tissue occurs around fat deposits and possibly as sheaths round the collagen fibre bundles (Dempsey, 1946). It is extremely difficult to isolate these three different tissues from skins. Tissues which stain similarly can be isolated fairly readily from other parts of the animal body: elastic fibres from the ligamentum nuchae (Vandegrift & Gies, 1901) and reticular tissue from lymph nodes (Bate-Smith, 1947) and from the large fat deposits (Maximow & Bloom, 1935; Dempsey, 1946), and some studies have been made on them. Before these tissues can be assumed to be identical with those occurring in skin, however, more conclusive evidence than staining reactions is required.

The aim of the present investigation was to determine what major differences in composition existed between the three main types of connective tissue, and to obtain information as to whether similar tissue preparations from different sources had the same composition.

EXPERIMENTAL

Analytical methods

Total N, amide N, amino N and titration curves were determined as described by Bowes & Kenten (1948*a*).

Preparation of samples

Ox-hide and alkali-treated sheepskin collagen were prepared as previously described (Bowes & Kenten, 1948*a*, *b*). Reticular tissue was prepared from lymph nodes and from the adipose tissue of ox. The wet lymph nodes were sliced, extracted with 5% (w/v) NaCl, washed and macerated with many changes of 30% ethanol in water. The fibrous mass was

dehydrated in 98% ethanol, extracted with light petroleum at 35° and air dried. Sheets of tissue from the fat deposit of an ox were mechanically freed from fat, extracted with light petroleum at 35°, washed with water and ethanol and dried in air.

The total N of the reticular tissues was rather lower than that of the collagen or elastin. The amide N was higher than that of elastin or of alkali-treated sheepskin collagen, and of the same order as ox-hide collagen (Table 1).

Table 1. *Analyses of connective tissues*

(Moisture and ash-free basis.)

Preparation	Total N (%)	Amide N (mmol./g.)	Amino N (mmol./g.)
Collagen (ox-hide)	18·6	0·66	0·46
Collagen (sheepskin, alkali-treated)	17·3	0·35	0·35
Elastin (ligamentum nuchae)	17·0	0·20	0·07
Reticular tissue (adipose tissue)	16·1	0·50	—
Reticular tissue (lymph nodes)	16·1	0·58	—

Elastin was prepared from the ligamentum nuchae of a freshly slaughtered ox. The tissue was freed from adhering matter, cut into approximately 0·5 cm. cubes, extracted with 10% (w/v) NaCl, washed free from salt, dehydrated with acetone and extracted with light petroleum (b.p. 40–60°) in a Soxhlet for 12 hr. The air-dried material (180 g.) was a pale yellow colour (N content 16·3%, moisture- and ash-free basis). To remove collagen, 168 g. of this material were autoclaved with 500 ml. water for 2 hr. at 120°. The supernatant liquor and the hot-water washings were combined and the total N extracted determined (see Table 2). (Increasing the time of autoclaving to 3 hr. caused no appreciable increase in the N extracted.) The autoclaved material was given eight further extractions of 1 hr. duration with 350 ml. portions of boiling water, and the total N removed in these extractions determined (Table 2). The elastin was dehydrated with acetone, again extracted with light petroleum and air dried (132 g.). Assuming that the residual material is elastin, the ligamentum nuchae after extraction with salt and removal of fat contained about 78% elastin and 22% collagen. This is in agreement with the value of 81% elastin found by Vandegrift & Gies (1901) and later by Lowry, Gilligan & Katersky (1941) for elephant ligamentum nuchae.

Table 2. *Soluble nitrogen extracted from ligamentum nuchae*

Extractions	Nitrogen extracted (% dry tissue)	(% total N)
During autoclaving	2·00	14·92
Subsequent extractions with water:		
First four	0·81	6·05
Fifth	0·066	0·49
Sixth	0·035	0·26
Seventh	0·008	0·06
Eighth	0·010	0·08
Total	4·921	21·86

The value for the total N of elastin (Table 1) is in agreement with previously determined values (Zoja (1897), 16·96; Richards & Gies (1902), 16·87; Stein & Miller (1938), 17·1%). The amide N, however, is appreciably lower than the two other values reported in the literature (Horbaczewski (1882), 0·41; Stein & Miller (1938), 0·38 mmol./g.); this is probably due to the fact that under the conditions used in earlier methods N, other than amide N, was returned as NH_3.

RESULTS

Titration curve of elastin

The titration curve of elastin with hydrochloric acid in the presence of 0·5 M-sodium chloride is given in Fig. 1, together with the corresponding curve for ox-hide collagen. The elastin shows an isoelectric point in the neighbourhood of pH 6. The curve shows a tendency to flatten between pH 2·0 and 1·5, but as the pH decreases further the amount of acid bound begins to increase again. This may be due to titration of fairly strong acid groups or of some weakly basic groups. The soluble nitrogen of the final solutions at pH values below 2·0 was only of the order of 0·5 mg./g. elastin, indicating that there was little solubilization of the protein. The curve indicates the presence of at least 0·2 mmol. basic groups per g. elastin.

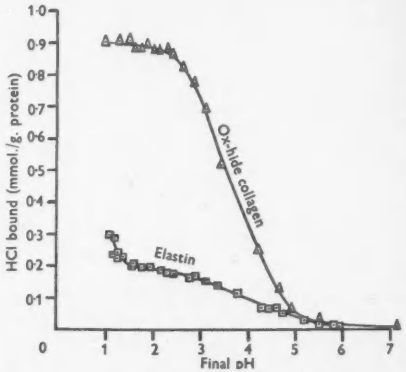

Fig. 1. Titration curves of elastin and ox-hide collagen in the presence of 0·5 M-NaCl.

Paper partition chromatography of samples

The technique described by Consden, Gordon & Martin (1944) was followed. The air-dry tissue (10±1 mg.) was hydrolysed with 0·5–1·0 ml. 6N-HCl at 100° for 24–30 hr. The hydrolysate was concentrated three times *in vacuo*, dissolved in 0·2 ml. water, and 10 µl. applied to a large sheet of Whatman no. 1 filter paper. The chromatogram was run in one direction with *s*-collidine in an atmosphere of diethylamine and HCN, and in the other direction with phenol in an atmosphere of NH_3 and coal gas. The chromatograms were examined immediately after spraying with ninhydrin, and

an estimation of the amino-acids present made from the size and colour intensity of the spots (Table 3). The strongest spots were arbitrarily assigned the value 9 and those just visible in a good light 1. Proline and hydroxyproline, which give yellow and orange colours respectively, were assessed as strong (S), medium (M) and weak (W).

Qualitative analyses by two-dimensional paper chromatographs of the reticular membrane from fat deposits and of elastin from ligamentum nuchae have been reported (Jordan Lloyd, 1946). In general the results agree, except that Jordan Lloyd reports the presence of hydroxyproline in elastin; this may be due to incomplete removal of the collagen. This author also reports that reticular tissue from fat deposits contains no tyrosine or hydroxylysine, whereas these were present in small amounts in our preparations. Consideration of the figures in Table 3 shows that elastin differs from collagen and both reticular tissue is converted to gelatin and a loose powdery substance which he terms 'reticulin' and which only slowly passes into solution. Both tissues lost approximately 50% in weight, but while the chromatogram of the lymph-node tissue was not appreciably changed, that of the reticular tissue from the fat deposits showed a definite decrease in hydroxyproline. This suggests that this reticular tissue contained some collagen, and, in confirmation, the aqueous extract obtained on autoclaving formed a gel, while a similar extract from lymph nodes did not (see also Bate-Smith, 1947). On autoclaving for 18 hr. at 105°, 73 mg. of the air-dry reticular tissue from the fat deposits gave a residue of only 6 mg. This residue resistant to autoclaving might be expected to be elastin, but the paper chromatogram showed that this was unlikely since it contained appreciably more of the di-

Table 3. *The amino-acid distribution in some preparations of connective tissues as indicated by the relative intensities of spots on paper chromatograms*

Material ...	Collagen			Reticular tissues					Collagen residues	
				Lymph nodes		Fat deposits				
Source ...		Alkali-treated sheep-skin	Elastin Ligamentum nuchae	Macerated	Auto-claved 4 hr.	Extracted with light petroleum	Auto-claved 3 hr.	Auto-claved 18 hr.	Ox-hide	Alkali-treated sheep-skin
Amino-acids:	Ox-hide									
Alanine	7	7	9	8	8	9	8	8	6	9
Glycine	9	9	9	9	8	9	9	8	6	9
Leucine, etc.*	5	6	9	9	9	7	7	9	9	8
Phenylalanine	2	2	3	3	3	2	2	2	3	3
Valine	5	5	8	5	5	6	6	7	7	7
Cysteic acid	1?	1?	1	1	1	1	1	1	?	1
Serine	3	3	2	5	4	5	4	4	4	4
Threonine	2	2	2	4	4	3	3	4	3	3
Tyrosine	1	1	2	3	3	1	2	1	2	2
Arginine	5	5	1	6	6	4	6	3	4	3
Histidine	2	2	—	2	2	1	1?	—	2	1
Hydroxylysine	2	2	—	1	1	2	2	—	—	—
Lysine	5	5	1	6	7	4	5	5	5	3
Aspartic acid	5	6	3	7	7	5	6	5	7	4
Glutamic acid	7	7	4	8	8	7	9	6	8	6
Proline	S	S	S	M	M	M	M	M	M	M
Hydroxyproline	S	S	—	M	M	M	W	Trace	—	—

* Leucine, etc., refers to a composite spot which may contain leucine, isoleucine, and methionine.

types of reticular tissue in containing less arginine, lysine, hydroxyproline and glutamic acid, rather less aspartic acid, and rather more valine. In addition, it differs from the reticular tissues in containing rather less serine and rather more proline. Collagen differs from the reticular tissues in containing more proline and hydroxyproline. The reticular tissue from the fat deposits contains rather less of the dicarboxylic and basic amino-acids than the lymph node tissue.

It was considered possible that the reticular tissues were contaminated with collagen. The tissues were autoclaved for 3 hr. at 105°. This should remove collagen; but according to Siegfried (1902) carboxylic and basic amino-acids than elastin from ligamentum nuchae. It is possible that it is identical with Siegfried's 'reticulin'. Similarly, the residues (11 mg.) obtained on twice autoclaving ox-hide (2 g.) for 6 hr. at 105° followed by boiling for 1–2 hr., and the residue (30 mg.) obtained by boiling 4·76 g. limed sheepskin with 100 ml. 0·01 N-sodium hydroxide for 15 min., followed by repeated extractions with boiling water, gave paper chromatograms distinguishable from that of elastin by the presence of histidine and larger amounts of the dicarboxylic and basic amino-acids. The two residues differed slightly from one another, particularly in respect to the dicarboxylic acids, and from lymph-node reticular

tissue in containing no hydroxyproline. The absence of this amino-acid indicated the complete removal of collagen.

DISCUSSION

It is of interest to compare values given in the literature for the amino-acid composition of elastin with the results of paper partition chromatographic examination of the present preparation of elastin (Table 4). The chromatogram indicates that the values reported for aspartic acid and alanine are low, and that serine is present in approximately the same amount as threonine. The low figures for leucine and isoleucine obtained by Graham, Waitkoff & Hier (1949), using microbiological methods, suggest that the 'leucine fraction' of Table 4 may contain amino-acids other than leucine and isoleucine in comparatively large amounts. No hydroxyproline was detected in the chromatogram of the present preparation, and although it may not be entirely absent, the value of 2% given by Stein & Miller (1938) is possibly too high and may be due to incomplete removal of collagen. The low value for the amino nitrogen is in agreement with the small amount of lysine detected by analysis and by paper chromatography. The titration curve indicates that at least 0·2 mmol./g. of basic groups must be present in elastin; the amino nitrogen (0·07 mmol./g.), together with 0·06 mmol./g. of arginine (Stein & Miller, 1938), accounts for 0·13 mmol./g., leaving 0·07 mmol. of base to be accounted for. Histidine was not detected by paper chromatography, and consequently is present, if at all, in very small amount; it seems likely, therefore, that the discrepancy between the titration curve and analysis is due to a low arginine value.

Table 4. *Composition of elastin*

	Amino-acid (g./100 g.)	Amino-acid (N as % protein N)	Relative intensities of spots on paper chromatograms
Glycine	29·4 (*a*)	32·3	9
	25·75 (*b*)	28·3	
Alanine	6·85 (*b*)	6·3	9
Leucine fraction	30·0 (*a*)	18·8	9*
	21·38 (*b*)	13·4	
	10·7 (*h*)	6·7	
Valine	13·5 (*a*)	9·5	8
	13·8 (*h*)	9·7	
Phenylalanine	3·89 (*b*)	1·9	3
	3·34 (*c*)	1·7	
	4·8 (*h*)	2·4	
Tryptophan	0·0 (*a*)	—	—
Serine	—	—	2
Threonine	2·7 (*g*)	1·9	2
	1·1 (*h*)	0·8	
Cystine	0·23 (*a*)	0·2	(Cysteic acid = 1)
	0·6 (*h*)	0·4	
Methionine	0·38 (*a*)	0·2	—
	0·03 (*h*)	—	
Proline	15·2 (*a*)	10·9	5
	15·6 (*h*)	11·2	
Hydroxyproline	2·0 (*a*)	1·3	0
Arginine	1·0 (*a*)	1·9	1
	0·3 (*f*)	0·6	
	1·1 (*h*)	2·1	
Histidine	0·0 (*a*)	0·0	0
	0·04 (*h*)	0·1	
Lysine	0·0 (*a*)	0·0	1
	0·5 (*h*)	0·6	
Hydroxylysine	—	—	0
Aspartic acid	0·0 (*a*)	0·0	3
	0·6 (*b*)	0·4	
Glutamic acid	2·7 (*b*)	1·5	4
	3·3 (*h*)	1·8	
Tyrosine	1·6 (*b*)	0·7	2
	0·25 (*d*)	0·1	
	0·34 (*e*)	0·2	
	1·4 (*h*)	0·6	

* Isoleucine is present.

(*a*) Stein & Miller (1938). (*b*) Abderhalden & Schittenhelm (1904). (*c*) Kapeller-Adler (1932). (*d*) Horbaczewski (1882). (*e*) Schwarz (1893). (*f*) Kossel & Kutscher (1898). (*g*) Brand & Kassel (1942). (*h*) Graham *et al.* (1949).

Taking the highest figure for the 'leucine fraction' (30%) it is possible to account for approximately 90% of the total nitrogen of elastin. Judging from the chromatogram and the titration curve, about half this deficiency is due to the presence of more serine, arginine and aspartic acid than the present analytical figures suggest, leaving 5% still to be accounted for. It may be noted that Stein & Miller (1938) find that the sum of sulphur in methionine (0·08%), cystine (0·06%) and occurring as $SO_4^=$ (0·02%) agrees closely with the total sulphur of elastin (0·16%).

Comparison of the chromatograms suggests that elastin and reticular tissue from different sources may differ appreciably in composition. The residues from the exhaustive extraction of ox-hide, sheepskin and reticular tissue from fat deposits, which on the reported properties of collagen, elastin and reticular tissue, should consist mainly of elastin, do not give chromatograms similar to that of elastin from ligamentum nuchae, but, with the exception of the absence of hydroxyproline, give chromatograms more nearly resembling that of lymph-node reticular tissue. It appears, therefore, that the elastic fibres of skin are either appreciably different in composition from those of the ligamentum nuchae, or that they are less resistant to boiling water, and the residues obtained from skin consist of some other protein or proteins resembling reticular tissue in composition.

In view of the suggestions that have been made regarding the possible identity of reticulin and precollagen (Heringa & Weidinger, 1942) it is of interest

to note that with the exception of a lower proline and hydroxyproline content, the amino-acid distribution of the collagen and reticular tissue preparations are similar.

SUMMARY

1. Collagen has been prepared from ox-hide, elastin from ligamentum nuchae and reticular tissue from lymph nodes and fat deposits. Total nitrogen, and amide nitrogen have been determined and paper chromatograms of the hydrolysates examined.

2. Examination of the titration curve and chromatogram of elastin indicates that several amino-acids have still to be determined and that the reported values for arginine and alanine are low.

3. Tissues from different sources which stain similarly may vary appreciably in composition. With the exception of a lower proline and hydroxyproline content, the amino-acid distribution of the reticular tissues was similar to that of collagen.

4. Examination of chromatograms of the residues obtained by the exhaustive extraction of skin suggests that the elastic fibres of skin either differ from those of ligamentum nuchae with respect to amino-acid composition or are less resistant to hot water.

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A Differential Method for the Detection of Small Differences in Mobility of Colloids in Electrophoresis

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The determination of differences between the mobilities of two colloids by the standard technique of measuring the boundary displacements in two separate experiments necessitates the rigorous prevention or control of movements of the boundaries due to causes other than the migration of the colloid in the electric field. The method described here has the advantage of eliminating possible complications of that kind. Its main feature is the simultaneous observation of the boundaries of two components A and B, in an electrophoresis run, in which a layer of a solution of one component, A or B, is put on top of the mixture of A and B, the concentration of A or B being the same in both layers.

Two experiments are set up as shown in Fig. 1*a*, *b*. The boundaries are first brought into view (Fig. 1*c*) and, after the current has been started, are kept in position by compensation, i.e. by running buffer into the electrode vessel towards which the migration takes place. The distance between the two ascending boundaries is recorded at intervals. Experiments have also been made in which the supernatant liquid

on either side of the mixture is a solution of one of the components. In this case the experiment was set up as shown in Fig. 1*d*, *e*.

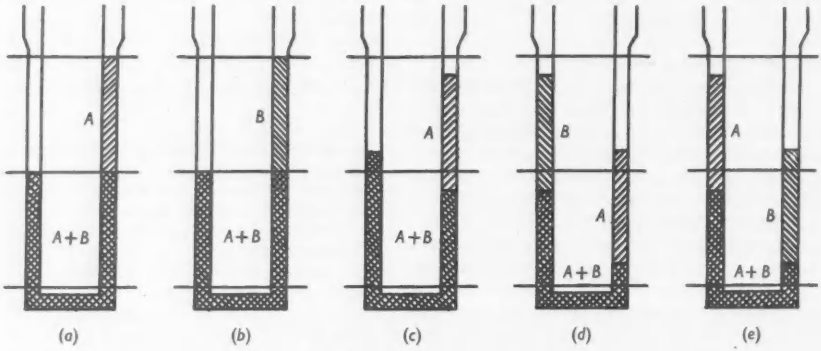

Fig. 1. Arrangement of solutions in electrophoresis cell. Ascending, right; descending, left.

THEORETICAL

In the following discussion colloids only are considered which give gravitationally stable boundaries in ordinary runs in the same buffer. Two kinds of interference may arise. (*a*) When solutions equilibrated by dialysis are used, δ-boundaries appear below both the lower and the upper ascending boundaries. The velocity of migration of the lower ascending boundary is bound to change when this boundary and the δ-boundary of the solution in the upper layer cross. The δ-boundaries can be almost completely eliminated if the solutions containing *A* or *B* are prepared from those which establish themselves, on preliminary electrophoresis of *A* and *B* respectively, below the ascending boundary ('adjusted solutions', Longsworth & MacInnes, 1940*a*; Svensson, 1946) if the same buffer is used as supernatant throughout. A solution prepared by dilution of an adjusted solution with the buffer is not identical with one recoverable from below the ascending boundary after an electrophoresis in which the colloid concentration had been the same as that of the diluted solution. One may expect the differences in the ionic concentrations between these two solutions to be small, and the δ-boundaries negligible, if the change in migration velocity of the ascending boundary is approximately proportional to the change in colloid concentration, within the concentration range between the undiluted and diluted solutions. This range is greatest at low concentrations and it increases with the salt concentration of the buffer solution.

In an experiment as in Fig. 1*a*, *b* and *c* with 'adjusted solutions', the pattern on the descending side represents the electrophoresis of the mixture of the two components in which the ε-boundary is absent. Usually the conductivity of the 'adjusted solution' is less than that of the buffer solution, and the migration velocity of the colloid, the current being kept constant, increases with increasing colloid concentration. This increase is greater at higher colloid concentration since the transport number of the colloid also increases. Thus on dilution of an 'adjusted solution' with buffer, the migration velocity of the colloid will be slightly greater than in a solution adjusted at the concentration of the diluted solution. A gravitationally unstable region necessarily forms at the boundary. Judging by the shape of the boundaries in experiments in which diluted solutions were prepared from 'adjusted solutions' containing 1–1·5% protein, this boundary disturbance appears to be negligible for albumin in phosphate at $\mu = 0{\cdot}1$ and oxyhaemoglobin (HbO_2) in phosphate at $\mu = 0{\cdot}05$ (μ = ionic strength).

(*b*) According to the moving boundary equation (Svensson, 1943, 1946; Dole, 1945) the concentrations of all ions change at every boundary. Since in the present experiments the colloid concentration in the upper compartment was chosen to be the same as in the mixture in the bottom compartments, 'false' boundaries (as defined by Svensson, 1946, p. 14; i.e. boundaries where no ion disappears) due to changes in concentration of that component which is present on both sides of the lower ascending boundary, must be expected ahead of this boundary if the faster, and behind it if the slower of the two components is placed above the mixture. In at least one of these cases a gravitationally unstable region is bound to form at the lower ascending boundary. Thus, if the faster component *A* is placed above the mixture of *A* and *B*, the boundaries will be gravitationally stable if $V_{Am} > V_A$ (V_A = migration velocity of *A* in unitary solution, such as in the solution between boundaries 1 and 1* of Fig. 2*a*; V_{Am} = migration velocity of *A* in the mixture), and a false boundary, 1*, will form ahead of 2 (Fig. 2*a*). If $V_{Am} < V_A$ the boundary region above 2 is gravitationally unstable and it will rise to a level above 1 (Fig. 2*b*, *c*). In both cases the change in the distance between the ascending boundary regions of *A* and *B* is less than it would be if no changes occurred in the concentration of *A* at the boundary of *B*. When *B* is placed above the mixture and if $V_{Bm} > V_B$ (V_B = migration velocity of *B* in unitary solution; V_{Bm} = migration velocity of *B* in the mixture), a gravitationally unstable region is probable near the boundary of *A*. It is conceivable that the material in this region flows off as soon as it separates, and that the migration velocity of the boundary of *A* is equal to V_{Am}. If $V_{Bm} < V_B$ (this is only likely if $V_{Am} < V_A$ and not if $V_{Am} > V_A$, since interaction, in the sense of specific attraction, between *A* and *B* would make the average migration velocities differ less, but not more), a false boundary will form below 2, and the change in the distance between the boundaries of *A* and *B* will also

appear reduced here (Fig. 2*d*). It is mainly because of these boundary anomalies that the experiments must be carried out at low colloid concentrations. It does not seem possible to derive the upper limit of colloid concentrations permissible by calculation without making assumptions about the mobilities of the components in the mixture. This limit certainly depends on the relative difference in mobility of the two components.

in migration velocity between the slower component and the complex, i.e.

$$d = V_B - V_{Am} = \tfrac{1}{2}(V_B - V_A) \quad \text{if} \quad V_{Am} = V_{Bm} = \tfrac{1}{2}(V_A + V_B).$$

In an actual experiment the gravitationally unstable region would mix, at least partially; consequently $d > \frac{1}{2}(V_B - V_A)$.

It can be concluded that at infinitely low colloid concentration, and in the absence of interaction between the

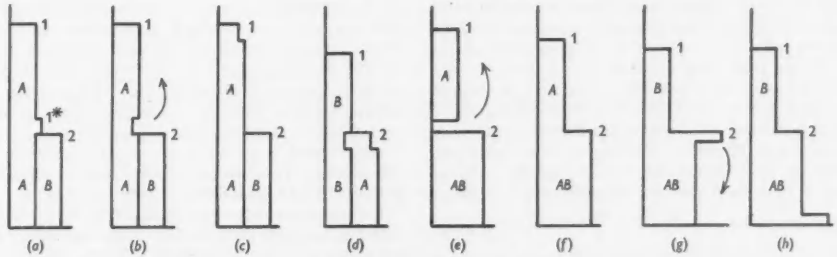

Fig. 2. False boundaries in the differential method. Abscissae = concentrations of colloid; ordinates = vertical distances in electrophoresis cell.

Assuming that V_{Am}/V_A does not differ appreciably from unity and disregarding, for the present argument, the boundary disturbances discussed above, the difference of the changes after unit time in the distances between the two ascending boundaries, in experiments as in Fig. 1*a*, *b*, is equal to the sum of the absolute migrations, and is given by

$$d = (V_A - V_{Bm}) - (V_B - V_{Am}).$$

Introducing ϵ by $\dfrac{V_{Am} - V_{Bm}}{V_{Am}} = \dfrac{V_A - V_B}{V_A}(1 - \epsilon)$,

$$d = V_A - V_B + \frac{V_{Am}}{V_A}(V_A - V_B)(1 - \epsilon),$$

and the difference in migration velocity of A and B

$$V_A - V_B = \frac{d}{1 + \dfrac{V_{Am}}{V_A}(1 - \epsilon)}.$$

If $V_B/V_A = V_{Bm}/V_{Am}$, which is probable if no interaction occurs between the two components, and if the colloid concentration is sufficiently low for the differences between the effects of ionic strength on the mobility of each component to be negligible, then

$$\epsilon = 0 \quad \text{and} \quad V_A - V_B = \frac{d}{1 + \dfrac{V_{Am}}{V_A}} = \sim\frac{d}{2};$$

if $V_{Am} - V_{Bm} < V_A - V_B$, indicating interaction between the components in the mixture, then $\epsilon > 0$ and $V_A - V_B > \frac{1}{2}d$. If $V_{Am} - V_{Bm} = 0$, i.e. maximum interaction, the mixture of the two components would behave as one component and gravitationally unstable regions would form near the lower of the two boundaries. Imagining the transfer of the unstable regions to stable positions to occur without mixing with the liquid through which they pass, the events may schematically be represented as in Fig. 2*e–h*. On migration, with the faster component in the upper layer, the distance between the ascending boundaries would not change, while with the slower component in the upper layer the change in the distance in unit time would be equal to the difference

two components, the difference between the velocities of the two components is very nearly equal to the change per unit time in the distance between the two ascending boundaries. In this case V_{Am}/V_A involves only the ratio of cross-sections at the levels of the boundaries. At finite colloid concentrations the difference in migration velocity of the components is always greater than this, as shown by the analysis of the possible combinations of relative values for V_A, V_{Am}, V_B and V_{Bm}.

Thus a limitation of the method is indicated on theoretical grounds by the finding that, on account of boundary anomalies and possible interaction between the components, a minimum value only for the difference in mobility can be obtained. The difference in mobility, $u_A - u_B$, corrected to infinitely low colloid concentration (Hoch, 1948*b*), is given by

$$u_A - u_B = \frac{\kappa q}{I}\left(\frac{V_A}{1 + K_A C_A} - \frac{V_B}{1 + K_B C_B}\right),$$

where κ = conductivity of the buffer solution, q = area of cross-section at the position of the upper boundary, I = current, K_A and K_B = coefficients defined by $K_i = \dfrac{V_{ai} - V_i}{C_i V_i}$ (V_{ai} = actual migration velocity of ascending boundary, V_i = migration velocity of component i at infinitely low concentration in the supernatant buffer, C_i = concentration of component i in g./100 ml.). If the concentrations C_A and C_B are chosen so that $K_A C_A$ and $K_B C_B$ are equal, then

$$(u_A - u_B) = \frac{\kappa q}{I(1 + K_A C_A)}(V_A - V_B).$$

Substituting for $(V_A - V_B)$,

$$(u_A - u_B)_{\text{min.}} = \frac{\kappa q}{I(1 + K_A C_A)} \cdot \frac{d}{1 + \dfrac{V_{Am}}{V_A}(1 - \epsilon)}. \qquad (1)$$

Although an estimate of possible deviations of the expression for $(u_A - u_B)_{\text{min.}}$ from the true difference in mobility cannot be made, a qualitative argument may provide a means of

limiting the range of uncertainty. It can be shown (Hoch, 1948*a*) that the boundary anomalies become considerable when the relative change in migration velocity of an ion across a protein boundary is of the same magnitude as the relative difference in the mobilities of that ion and of the protein. If, for example, the mobilities differ by five times this amount, the concentration changes across the protein boundary may be disregarded, and the uncertainty in the difference in mobility as calculated from equation (1) can be limited to the effects of interaction. Thus, for small relative differences in mobility, about as great as the relative change in migration velocity of ions across the protein boundary, a value of five times that obtained by equation (1) may arbitrarily be taken as the probable upper limit for the true value, assuming little or no interaction. With comparatively large relative differences in mobility, the boundary anomalies may be neglected. Favourable working conditions are therefore near the isoelectric point and at high salt concentrations, so that the value for the difference in mobility may be bracketed between $\frac{\kappa q}{I}\frac{d}{2}$ and $\frac{\kappa q}{I}2d$ if there is interaction between the components. In view of these uncertainties the following approximate form of equation (1) was used in the present paper:

$$(u_A - u_B)_{\min.} = \frac{\kappa_B q}{I}\frac{d}{2}, \qquad (2)$$

where κ_B = conductivity of the buffer solution.

The method appears to be useful for the purpose of establishing the identity of two colloids. Here K_A and K_B are assumed equal and experiments (*a*) and (*b*) of Fig. 1 must give identical changes in the relative positions of the boundaries if $C_A = C_B$. However, identical changes might be obtained also if $\frac{V_A}{1+K_AC_A} = \frac{V_B}{1+K_BC_B}$ for $V_A \neq V_B$ at a particular concentration of A (=that of B). But the uncertainty should be resolvable on carrying out the experiment at different salt or colloid concentrations. For example, if $K_A = 0{\cdot}06$ and $K_B = 0{\cdot}05$, $C_A = C_B = 0{\cdot}3$ g./100 ml. V_A/V_B could be 1·003, although no difference would be found. But at $C_A = C_B = 0{\cdot}06$ g./100 ml. a relative difference of 0·3 % in the migration velocities should be detected.

The differential method was applied to the comparison of serum and urine albumins, and to that of human and rabbit oxyhaemoglobin.

EXPERIMENTAL

The experiments were made in the Tiselius apparatus at 0·1°. Three different medium-size (11 ml.) cells with two middle sections and the cylindrical lens schlieren optical system (Thovert, 1914; Philpot, 1938; Svensson, 1939) were used.

Materials. Fresh blood and urine were obtained from nephritis patients with albuminuria. The solutions of HbO_2 were prepared as follows: 1–2 ml. of packed red cells were washed 6–8 times with 2 vol. of a mixture consisting of 0·85 % NaCl (2 vol.) and Na phosphate buffer, pH 8, μ, 0·2 (1 vol.). The cells were then lysed with 2 vol. of water and the solution centrifuged. On addition of pH 8 phosphate buffer to the clear supernatant a precipitate formed which was centrifuged down. This precipitation eliminated the fast component and the slight turbidity which was previously observed by Stern, Reiner & Silber (1945). The supernatant liquid was clear in every preparation.

Solutions of the sera were made up in Na phosphate buffers, pH 7·7 or 8·0, and ionic strength 0·1; those of HbO_2 in Na phosphate buffers, pH 7·1 and 8·0. The pH 7·1 buffer was that used by Andersch, Wilson & Menten (1944). The ionic strength of the pH 8 buffer used for the electrophoresis of HbO_2 was 0·05.

The adjusted solutions of albumin were prepared by the electrophoresis of serum at concentrations yielding albumin solutions containing 0·4–1·5 g. protein /100 ml. The HbO_2 solutions were electrolysed at concentrations of about 1 g./100 ml. The preparatory runs were continued until two-thirds or the whole ascending limb was occupied by the adjusted solution. The adjusted solutions were appropriately diluted with buffer and equal volumes of each component, A and B, were mixed to give the solution for the bottom layer; smaller portions were diluted with an equal volume of buffer to give the solutions for the upper layer.

The concentrations of albumin and of HbO_2 were determined by micro-Kjeldahl. HbO_2 was also determined by measuring the light absorption of HbO_2 and of HbCO in dilute NH_3 (2·5 ml. of NH_3 sp.gr. 0·880/100 ml.) in the visible region.

Observation of boundaries. The boundaries were brought into view by compensation and the patterns, which were obtained with a diagonal wire in all differential experiments, were photographed at frequent intervals during the run. The use of a wire (Svensson, 1939) facilitates the location of the line forming the pattern and this pattern is not subject to distortion if unevenly exposed (Svensson, 1939). The enlarged patterns were traced on graph paper, and the position of the centroidal ordinates of the peaks (Longsworth, 1943) was determined by making fifteen to thirty readings, spaced at equal distances, on each boundary. The following form of the equation for the centroidal ordinate ($\bar{x}$) was found convenient for use without a calculating machine:

$$\bar{x} = \frac{\Sigma xy}{\Sigma y} = \frac{\Sigma\,(y_i - y_{-i})\mid x_i \mid}{2\Sigma y_i - \Sigma\,(y_i - y_{-i}) + y_0},$$

where y_i and y_{-i} = ordinates at symmetrical positions on both sides of a working zero near the expected position of the centroidal ordinate; y_0 = ordinate at the working zero; x_i = abscissae.

RESULTS

(*a*) *Albumin.* Table 1 gives the details of experiments with serum and urine albumins in three cases of nephritis, and Fig. 3 shows the plot of the distance between the ascending boundaries against time in cases 1 and 3. The minimum difference in mobility calculated from equation (2) was about $0{\cdot}03 \times 10^{-5}$, i.e. 0·5 % of the mobility of albumin, in two cases. In case 3 the difference in mobility was much greater, but the urine had been concentrated to a quarter of its volume before dialysis and this may have altered the mobility of the urine albumin.

It appears from Fig. 3 that it might be possible to detect an even smaller difference in migration velocity of two albumin boundaries than that found in case 1. The interpretation of such a small difference is difficult, apart from the interference by the

Table 1. *Comparison of electrophoretic mobilities of serum and urine albumins*

Case no.	(1*)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)	(11)
1	6·0	30	1·0	8·0	*U* 0·11 *S* 0·11	6·3	0·053 (±0·02)	400	0·2	0·03	*U*
2	5·6	123	0·5	7·7	*U* 0·18† *S* 0·22	5·7	0·04 (±0·04)	400	0·2	0·03	*U*
3	7·0	200	0·1	7·7	*U* 0·16 *S* 0·17	5·7	0·76 (±0·04)	520	2·4	0·42	*U*

* Column designation:

(1) Serum proteins, g./100 ml.
(2) Non-protein nitrogen, mg./100 ml.
(3) Urine protein, g./100 ml.
(4) pH of phosphate buffer; $\mu = 0{\cdot}1$.
(5) Albumin concentration in upper layer, g./100 ml.; *U* = urine albumin, *S* = serum albumin.
(6) Potential gradient, V. cm.$^{-1}$.
(7) Total change in distance between ascending boundaries, cm. (approximate error range).
(8) Total time, min.
(9) Difference in migration velocity of boundaries, 10^{-5} cm.sec.$^{-1}$.
(10) Minimum difference in mobility of components, from equation (2), 10^{-5} cm.^{2} V.$^{-1}$sec.$^{-1}$.
(11) Faster component.

† Concentrations estimated from refractive areas in this case only.

boundary anomalies discussed above, since it could also be produced by a change in pH of about 0·02 unit in one of the solutions.

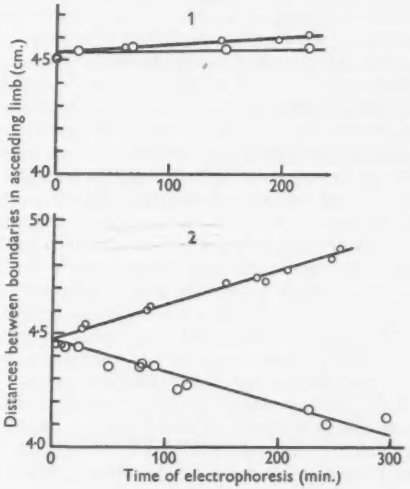

Fig. 3. Diagrams obtained by the differential method: albumins: smaller circles for the runs with the urine albumin in the upper layer. 1 = case no. 1 of Table 1; phosphate buffer, pH 8; μ, 0·1; 6·3 V./cm. 2 = case no. 3; phosphate buffer, pH 7·7; μ, 0·1; 5·7 V./cm.

In the three cases studied, the mean mobility of the urine albumin was the same or greater than that of the serum albumin from the same patient. It is necessary to speak of mean mobilities only, since both the serum and the urine albumin peaks proved composite in the preliminary runs as shown in Fig. 4. Serum albumin has previously been shown to be inhomogeneous at pH 8 (Blix, Tiselius & Svensson, 1941; Hoch & Morris, 1945; Armstrong, Budka & Morrison, 1947; Hoch, 1948*b*).

(*b*) *Haemoglobin.* In Table 2 and Fig. 5 are recorded the data of the comparison of the mobilities of oxyhaemoglobins from four different rabbits with the oxyhaemoglobin from a normal human adult. Rabbit oxyhaemoglobin had a smaller anodic mobility in all cases. There was no evidence of a difference between any of the normal rabbit oxyhaemoglobins.

Fig. 6 shows the patterns obtained in Exp. 3 of Table 2. The slowness of migration at pH 7·1 did not make the preparation of adjusted solutions practicable, and solutions equilibrated by dialysis were used. This was thought justifiable on account of the high ionic strength of the buffer and the small charge of the oxyhaemoglobin near its isoelectric point. The experiment was made at comparatively high protein concentration (0·5 g./100 ml.) so that boundary disturbances could be more readily observed. In both experiments set up as in Fig. 1*d*, *e*, the boundaries of the solutions of the single components did not appear to be disturbed by the formation of gravitationally unstable regions, but the shape of the curves deviated markedly from that of the Gaussian error curve. In the absence of boundary anomalies the lines relating the changes with time of the distances between the two ascending and the two descending boundaries should run parallel. That they diverged slightly (Fig. 5, no. 2) may have been due to the effects of the boundary anomalies.

Fig. 7 shows some patterns from Exp. 2 of Table 2, made at an unusually low protein concentration of 0·05 g./100 ml. The graph of the distances between the boundaries is seen in Fig. 5, no. 1.

If identity of two substances is suspected, the experiment may be carried out with solutions which

Table 2. *Comparison of electrophoretic mobilities of oxyhaemoglobins*

Exp. no.	(1)*	(2)	(3)	(4)	(5)	(6)	(7)	(8)
1	8·0	*H* 0·66 *R* 0·69	14	Asc. 1·01	300	5·6	0·4	*H*
2	8·0	*H* 0·05 *R* 0·05	10	Asc. 0·49 Desc. 0·49	220 220	3·7 3·7	0·37	*H*
3†	7·1	*H* 0·5 *R* 0·5	8·7	Asc. 1·02 Desc. 1·04	600 600	2·83 2·89	0·33	*H*
4†	8·0	$H_{(A)}$ 0·47 $H_{(B)}$ 0·47	14	Asc. 0·00	280	0	0	—

* Column designation:

(1) pH of phosphate buffer; $\mu = 0{\cdot}05$ at pH 8, $\mu = 0{\cdot}08$ at pH 7·1.
(2) Oxyhaemoglobin concentration in upper layer, g./100 ml.; *H* = human HbO_2, *R* = rabbit HbO_2; $H_{(A)}$ and $H_{(B)}$ = oxyhaemoglobins from two normal subjects.
(3) Potential gradient, V. cm.$^{-1}$.
(4) Total change in distance between the two ascending or the two descending boundaries, cm.
(5) Total time, min.
(6) Difference in migration velocity of boundaries, 10^{-5} cm.sec.$^{-1}$.
(7) Minimum difference in mobility of components, from equation (2), 10^{-5} cm.^{2} V.$^{-1}$sec.$^{-1}$.
(8) Faster component (anodic).

† In these experiments solutions equilibrated by dialysis were used.

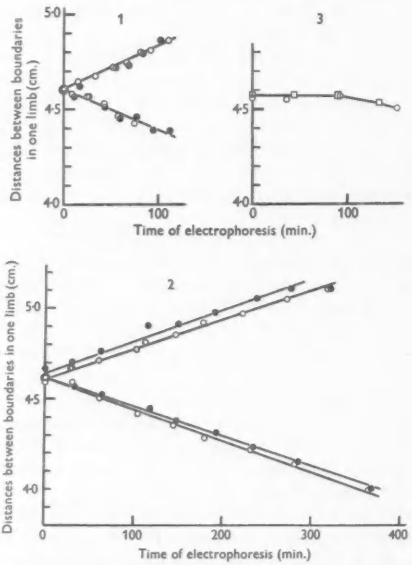

Fig. 5. Diagrams obtained by the differential method: oxyhaemoglobins. Exp. nos. 2–4 of Table 2. Hollow circles for the ascending, full circles for the descending limb; circles and squares in Exp. 4 for the ascending limb, with HbO_2 from subjects *A* and *B*, respectively, in the upper layer. The rising lines represent experiments in which the human, the falling lines those in which the rabbit HbO_2 was in the upper layer of the ascending limb. 1 = Exp. no. 2; phosphate buffer, pH 8; μ, 0·05; 10 V./cm.; HbO_2 concentration in upper layer, 0·05 g./100 ml. 2 = Exp. no. 3; phosphate, pH 7·1; μ, 0·08; 8·7 V./cm.; 0·5 g. HbO_2/100 ml. 3 = Exp. no. 4; phosphate buffer, pH 8; μ, 0·05; 14 V./cm.; 0·47 g. HbO_2/100 ml.

have not been subjected to preliminary runs for the preparation of adjusted solutions. Such an experiment is illustrated in Fig. 5, no. 3. The two adult human oxyhaemoglobins used were equilibrated simultaneously by dialysis against the same buffer solution. The migration was stopped before the δ-boundary of the solution, originally in the top compartment, reached the lower boundary. No difference was found in the relative positions of the two ascending boundaries at the end of the run, between Exp. (*a*) in which the oxyhaemoglobin from subject *A*, and Exp. (*b*) in which the oxyhaemoglobin from subject *B*, was placed in the top compartment.

The tests for homogeneity of the dialysed oxyhaemoglobin preparations are summarized in Table 3. The experiments on human oxyhaemoglobin showed a component of smaller anodic mobility apart from the main peak in every case. At pH 7·1 no δ- or ϵ-boundary was seen to separate, but these boundaries may have been covered by the small component. The preparations of rabbit oxyhaemoglobin did not show this component (Table 3, column 1; Fig. 8, nos. 1 and 2). The ratios N/E^{HbCO} of different preparations were reproducible to within $\pm 2{\cdot}5\,\%$, but those of human and rabbit carboxyhaemoglobin (HbCO) differed significantly (column 2). So did the ratios E^{HbCO}/E^{HbO_2} (column 3).

(*c*) *Mixtures of human and rabbit oxyhaemoglobins.* These experiments were made with the object of obtaining an independent estimate of the difference between the migration velocity of the faster component, in unitary solution, and that of the slower component in the mixture at low oxyhaemoglobin concentrations, and to study the effects of boundary anomalies at higher concentrations. The results are given in Table 4 and Fig. 8. The concentrations of human and rabbit oxyhaemoglobin were made equal

Table 3. *Tests for homogeneity of oxyhaemoglobin preparations*

Red cells from	(1) Electrophoresis refractive area of slow component (% of total area)	(2) $N \times 5{\cdot}95/E_{604}^{HbCO*}$ Mean (no. of samples)	(2) Range	(3) $E_{604}^{HbCO}/E_{604}^{HbO_2}$ Mean (no. of samples)	(3) Range
Human (three subjects)	3–5·5 (5 prep.)	1·018 (4)	0·985–1·036	1·225 (6)	1·214–1·234
Rabbit (three animals)	0·5 (1 prep.) or none detected (2 prep.)	1·050 (4)	1·029–1·065	1·209 (8)	1·200–1·217

* N content of horse HbCO = 16·8% (Chibnall, Rees & Williams, 1943). The conversion factor for the colorimetric estimation was obtained by standardization against spectrographic data (Hoch & Jope, 1947, unpublished experiments).

Table 4. *Electrophoresis of mixtures of human and rabbit oxyhaemoglobins of known composition*

Exp. no.	Concentration of total HbO_2 in adjusted solution (g./100 ml.)	Proportion of human HbO_2 in terms of N content: Taken (%)	Found (%)	Difference in mobility* of components (10^{-5} cm.^{2}V.$^{-1}$sec.$^{-1}$)	
1	0·5	48·4	30	0·28	—
2	0·53	48·9	35	0·23	Bad separation
3	0·6	48·7	35	0·31	—
4	0·67	48·9	45	0·29	—
5	0·89	48·9	45	0·20	Bad separation
6	1·1	48·9	50	0·24	—
7	1·87	48·3	60	0·25	—

* Mobility calculated using potential gradient in supernatant buffer.

with respect to the light absorption of the carboxyhaemoglobin, using the Ilford spectrum green filter no. 604. Assuming the refractive areas to be proportional to the nitrogen content, the proportion of the area under the faster of the two main peaks should be about 49% of the total area under the two main peaks, after allowing for the presence of 5% of the more slowly migrating component in the preparations of human oxyhaemoglobin, and for the difference in the contents of N/unit extinction (Table 3). The increase in concentration in the direction of migration of the human oxyhaemoglobin across the boundary of the rabbit oxyhaemoglobin in the buffer used was calculated to be appreciable at total concentrations of 0·5 g./100 ml. or greater, using assumptions about the similarity of the two components of oxyhaemoglobin such as specified for two albumins previously (Hoch, 1948*b*), and a value for $K = 0{\cdot}1$ ($\pm 0{\cdot}2$) for both oxyhaemoglobins. This value has been obtained from experiments analogous to that previously described for serum albumin in which layers of concentrated and diluted solutions were made to migrate simultaneously (Hoch, 1948*a*). As can be seen in Fig. 8 and Table 4, the ascending patterns did not show the expected excess of the faster component (human oxyhaemoglobin). At a total concentration of 1 g./100 ml. the apparent proportions were about equal. At a higher concentration the faster component appeared only slightly increased. But at low concentrations the apparent proportions were anomalous, showing too little of the faster component. The same patterns were produced with mixtures of oxyhaemoglobin from different rabbits with oxyhaemoglobin from the same human adult. The descending patterns of the dilute solutions (Fig. 8) indicate that the faster peak is abnormally large; however, the spreading of the boundaries and the poor separation did not permit a quantitative analysis.

These results might be explained by an interaction (Longsworth & MacInnes, 1942) between the two oxyhaemoglobins resulting in a smaller average migration velocity of the human oxyhaemoglobin in the presence of rabbit oxyhaemoglobin. On the ascending side, therefore, an increase in migration velocity of the faster component (human oxyhaemoglobin) at the boundary of the slower component necessitates a diminished concentration of the faster component above this boundary, as has been observed in the experiments. On the descending side the effect is reversed, in that the concentration of the slower component is reduced in the layer above the boundary of the faster component. This argument is formally equivalent to that used by Longsworth & MacInnes, based on the assumption of a complex formation. Further support for this explanation is the lack of variation in the difference between the migration velocities with the HbO_2 concentration, at constant current, in the ascending limb (Table 4). This difference should become smaller with increasing concentration if there is no interaction.

The values for the difference in mobility of the boundaries in Table 2 and those in Table 4 disagree by more than can be accounted for by experimental error of the differential method. The small variation with changing oxyhaemoglobin concentration in the

differential method suggests little interference by boundary anomalies. In the experiments with oxyhaemoglobin mixtures, no change in concentration of the faster component across the boundary of the slower component appears to occur on the ascending side at medium oxyhaemoglobin concentration, the proportions of the refractive areas being the same as those of the components in the mixture. In an experiment set up as in Fig. 1*a* at similar oxyhaemoglobin concentrations, a layer of human oxyhaemoglobin solution was put above the layer of the mixture, thus merely shifting the boundary of the faster component upwards by a distance equal to the length of one compartment; consequently on migration the change in the distance between the boundaries should have been identical with that obtained in the experiment with the mixture. This was not the case. The reasons for this discrepancy may be sought in the uncertainty of locating the boundaries in the experiments with the mixtures of oxyhaemoglobins. The boundaries are evidently non-symmetrical, and in more concentrated solutions indications of streaming can be observed at the slower peak (Fig. 8, no. 11) as the separation of the peaks becomes more complete. At the earlier stages of the run the partition line between the peaks cannot be drawn with the accuracy required, and at the later stages when this uncertainty diminishes it is the one-sided tailing off of the peaks which interferes. The tendency then is to exclude parts of the outer tail regions of the boundaries when locating the peaks, and accordingly the measured distance between them is too small. Although it appears that this error does not account for the whole of the discrepancy between the values in Tables 2 and 4, the remaining difference is too small to form a basis for any conclusions.

DISCUSSION

The boundary anomalies are at a minimum near the isoelectric point (Longsworth, Cannan & MacInnes, 1940) and they are probably negligible in an experiment with oxyhaemoglobins at pH 7·1, such as no. 3 in Table 2. They are presumably not of great importance in the experiments made with oxyhaemoglobins at pH 8, since similar differences between the mobilities were obtained at concentrations of 0·66 and 0·05 g./100 ml. The close agreement in these experiments between the values for d obtained from the ascending and descending sides, cannot be taken as indicating that the boundary anomalies could be neglected, since these may have been effective in the same direction in both limbs of the U-tube (Svensson, 1946). However consistent the experimental values may be for the minimum differences in mobility under varying conditions, for theoretical reasons it is not possible to obtain accurate values by the differential method, although the approximation may be good if the relative difference in mobility is not small, and if no interaction occurs between the components. The differential method might well be restricted for the present to establishing the identity of colloids, and to semi-quantitative statements on differences in mobility.

While the decision as to which of the components is the faster can be made with certainty from the observations by the differential method, such a decision may be misleading if the mobilities obtained in separate experiments are compared. This applies particularly when the mobilities are low, since slight deviations in the technique of reproducing the conditions in the electrode vessels may escape observation. In certain cases, e.g. in the comparison of serum and urine albumins, greater sensitivity could be obtained by the differential method than would be expected in absolute measurements of mobility.

The experiments described by Longsworth & MacInnes (1940*b*), who found that the mobilities of the urine albumins from nephrosis patients, in lithium-veronal buffer of pH 7·8, were consistently lower than those of the serum albumin, are not comparable with the present experiments on account of the different buffers used.

The anomalous proportions of refractive areas in mixtures of human and rabbit oxyhaemoglobins appear to indicate an interaction between these two components. Anomalous patterns with dilute solutions were found in four experiments, in which the components had been in contact for different lengths of time (1 hr., 12 hr., 3 days, 6 days), and therefore an explanation cannot be sought in a slow reaction between the components. Haemoglobin molecules have been shown to split into halves in dilute solution (MacCarthy & Popják, 1947; Gutfreund, 1948). If such a reaction occurred with both human and rabbit oxyhaemoglobin under the conditions of the present experiments, the possibility would have to be considered of the formation of mixed molecules of human and rabbit oxyhaemoglobin on recombination of the fragments. A mixed molecule would presumably migrate with a mobility intermediate between those of the components and three peaks should appear in the pattern, such as has been observed by Tiselius & Horsfall (1939) in experiments with haemocyanins; or if the mobilities were little different the boundary might appear as a single broad peak.

No useful information concerning interaction between the two oxyhaemoglobins was gained by electrolysing the mixture in phosphate buffer of pH 8 at the higher ionic strength of 0·2. The two components migrated as a single peak (Fig. 8, nos. 13–15). After dialysis of the same mixture against phosphate buffer of pH 8 and ionic strength 0·05, the patterns of Fig. 8, nos. 3 and 4 were obtained, showing two components as in the other experiments at this ionic strength.

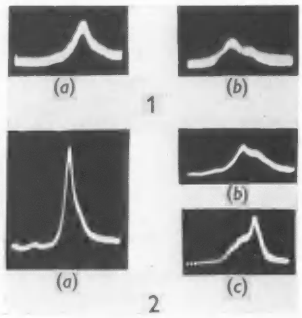

Fig. 4.

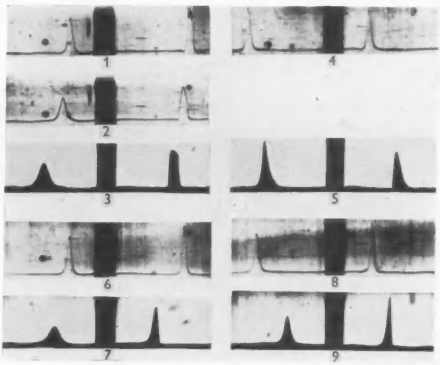

Fig. 6.

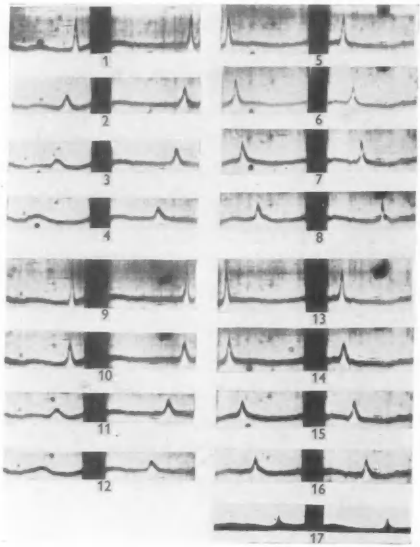

Fig. 7.

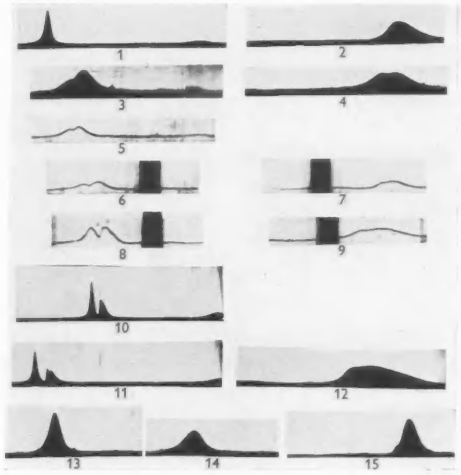

Fig. 8.

H. HOCH—A DIFFERENTIAL METHOD FOR THE DETECTION OF SMALL DIFFERENCES IN MOBILITY OF COLLOIDS IN ELECTROPHORESIS

SUMMARY

1. An electrophoretic technique is described in which the migration of two colloid boundaries, formed at a distance from each other, can be observed simultaneously. Under limiting conditions the change in the distance between the two boundaries is a measure of the difference in mobility of the two colloids. The differential method described is considered to be supplementary to and, in particular cases, more sensitive than that of comparing the mobilities in separate runs. The main field of application offers itself in the establishment of identity of colloids.

2. A modified formula for the calculation of the position of the centroidal ordinate is given.

3. The method was applied to the study of serum and urine albumins, and of human and rabbit oxyhaemoglobins. Serum and urine albumins from three cases of nephritis showed a minimum difference in mobility at pH 8 of about $0{\cdot}03 \times 10^{-5}$ in two cases, and of $0{\cdot}42 \times 10^{-5}$ in the third case. The urine albumin had the greater mobility. The minimum difference in the mobilities of human and rabbit oxyhaemoglobins was $0{\cdot}4 \times 10^{-5}$ at pH 8 and $0{\cdot}33 \times 10^{-5}$ at pH 7·1. The human oxyhaemoglobin had the higher anodic mobility. Oxyhaemoglobins from two normal adults showed no detectable difference in mobility.

4. Mixtures of human and rabbit oxyhaemoglobins of known composition gave patterns with two main peaks at pH 8 and ionic strength 0·05 in which the proportions of the refractive areas were abnormal. At pH 8 and ionic strength 0·2 the two components did not separate.

I wish to thank Prof. J. R. Marrack for encouragement and for criticism. I am greatly indebted to Prof. A. Tiselius for his interest and for hospitality in his laboratory, where I had the privilege of conducting part of this work, and to Docent H. Svensson, with whom I had the pleasure of many discussions of the problems involved. I should also like to thank Dr E. M. Jope for his help in connexion with the work on haemoglobin.

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EXPLANATION OF PLATE 3

Fig. 4. Electrophoretic patterns of serum and urine albumins in nephritis. Left, serum albumin; right, urine albumin. 1(*a*) and (*b*) ascending patterns from case 2 of Table 1. 2(*a*) and (*c*) ascending, (*b*) descending patterns from case 3 of Table 1. Ascending patterns after 7–9 hr., direction of migration towards the right; 2(*b*) after 3 hr., direction of migration towards the left; phosphate, pH 7·7; μ, 0·1; 9·5 V./cm.

Fig. 6. Electrophoretic patterns obtained by the differential method in Exp. 3 of Table 2. Run I, set up as in Fig. 1(*d*): nos. 1–3 ascending patterns, human HbO_2 in upper layer, after 60, 177 and 323 min. migration. Nos. 4 and 5 descending patterns, rabbit HbO_2 in upper layer, after 63 and 327 min. Run II, set up as in Fig. 1(*e*): nos. 6 and 7 ascending patterns, rabbit HbO_2 in upper layer, after 61 and 397 min.; nos. 8 and 9 descending patterns, human HbO_2 in upper layer, after 64 and 394 min. The direction of migration for the ascending patterns is towards the left, for the descending towards the right. In each run all four boundaries were of equal height and identical shape at the beginning of the experiment. Phosphate, pH 7·1; μ, 0·08; 8·7 V./cm.; HbO_2 concentration in upper layer, 0·5 g./100 ml.

Fig. 7. Electrophoretic patterns obtained by the differential method in Exp. 2 of Table 2. I: nos. 1–4 ascending patterns, human HbO_2 in upper layer, after 0, 33, 66 and 132 min. migration. Nos. 5–8 descending patterns, rabbit HbO_2 in upper layer, after 0, 36, 69 and 135 min. II: nos. 9–12 ascending patterns, rabbit HbO_2 in upper layer, after 0, 9, 58 and 111 min. Nos. 13–17 descending patterns,

human HbO_2 in upper layer, after 0, 12, 60, 113 and 185 min. The direction of migration for the ascending patterns is towards the left, for the descending towards the right. Phosphate, pH 8; μ, 0·05; 10 V./cm.; HbO_2 concentration in upper layer, 0·05 g./100 ml.

Fig. 8. Electrophoretic patterns of oxyhaemoglobins. All experiments, with the exception of nos. 13–15, in phosphate, pH 8; μ, 0·05; 14 V./cm. Nos. 1 and 2 ascending and descending patterns of rabbit HbO_2, concentration = 1·35 g./100 ml., after 430 and 435 min. Nos. 3–15 patterns of mixtures 1:1 of human and rabbit HbO_2. Left, ascending limb; right, descending limb. Nos. 3 and 4 = Exp. 1 of Table 4, after 320 and 322 min.; no. 5 = Exp. 2, after 299 min.; nos. 6 and 7 = Exp. 3, after 321 and 209 min.; nos. 8 and 9 = Exp. 6, after 382 and 385 min.; nos. 10–12 = Exp. 7, after 243, 360 and 364 min.; nos. 13 and 14 ascending patterns, no. 15 descending pattern of a mixture 1:1 of human and rabbit HbO_2 in phosphate pH 8, μ = 0·2; nos. 13 and 15 after 443 min. at 7 V./cm., then 160 min. at 6 V./cm.; no. 14 after 443 min. at 7 V./cm., then 173 min. at 6 V./cm., then 815 min. at 4 V./cm. Total distance migrated about 9 cm.

Chromatographic Studies of Nucleic Acids

1. A TECHNIQUE FOR THE IDENTIFICATION AND ESTIMATION OF PURINE AND PYRIMIDINE BASES, NUCLEOSIDES AND RELATED SUBSTANCES

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Until recently the isolation and identification of purine and pyrimidine bases, ribosides and nucleotides in mixtures such as nucleic acid hydrolysates were only possible by purely chemical methods of separation, necessitating the use of very large quantities (often 100 g. or more) of starting materials (Levene & Bass, 1931). Such methods are tedious, very limited in application and far from quantitative. However, the development of partition chromatography on paper (Consden, Gordon & Martin, 1944) has afforded the possibility of the separation of mixtures of purine and pyrimidine derivatives in minute amounts. The successful application of this method depends upon the location on the paper chromatogram of the areas occupied by the various substances. Once located, these areas may be cut out, extracted in a suitable solvent, and the substances estimated by ultraviolet spectrophotometry, making use of their characteristic absorption bands in the region of 260 mμ. Two methods of detecting purine and pyrimidine derivatives have been described recently. Hotchkiss (1948) cut the paper into transverse strips, 5–30 mm. wide, extracted each of these in water and, using a Beckman photoelectric spectrophotometer, determined for each extract the absorption in the ultraviolet region near 260 mμ. This method is time-consuming, does not allow of a very high resolution of adjacent spots, and for practical reasons is obviously inapplicable to two-dimensional chromatograms. In a second method, described by Vischer & Chargaff (1948*a*), the purines and the pyrimidines are converted on the chromatogram to mercury complexes. Excess mercury is removed and the paper is treated to convert the mercury in the complex into mercuric sulphide, which being black indicates the position of the spots. While this technique allows the detection of as little as 5 μg. of the free bases in individual spots, it has serious limitations. The method is not rapid, involving prolonged treatment of the paper in the wet state with several reagents and as described is limited to the bases. Furthermore, during the conversion of the mercury complexes into mercuric sulphide, the bases are lost so that for quantitative analysis parallel untreated strips of the chromatogram have to be used. It is thus impossible to use this technique for quantitative two-dimensional chromatograms.

To avoid these difficulties we have developed a simple and rapid method which will detect in a chromatogram all substances having a strong absorption in the ultraviolet near 260 mμ. Such substances, of course, include all purine and pyrimidine derivatives, as well as many other compounds of biological interest. The basis of this method, which has been reported briefly elsewhere (Markham & Smith, 1949) is a simple photographic method for the detection of absorbing substances.

METHODS

(*a*) *Location of the spots*

As all purine and pyrimidine derivatives have an intense ultraviolet absorption in the neighbourhood of 260 mμ. it is natural to look for methods suitable for exploiting this characteristic. Filter paper happens to be reasonably

transparent in this region, so it is possible to locate the spots of absorbing substances merely by making a contact print by filtered ultraviolet light on to sensitized paper through the dried paper chromatogram.

The source of ultraviolet light used in these experiments was a Hanovia 'Homesun' lamp, a 450 W., 200 V., mercury arc lamp. This lamp has a high emission at 253·7 mμ., a wave length which, though not ideal, is satisfactory for most purposes. In order to make the method as sensitive as possible it is desirable to use filters in order to obtain light of this wavelength as nearly monochromatic as possible, particularly as photographic materials are very sensitive to the longer wavelength ultraviolet light emitted by this lamp. Several filters are available for this purpose, but for convenience a liquid filter containing $CoSO_4$ and $NiSO_4$,

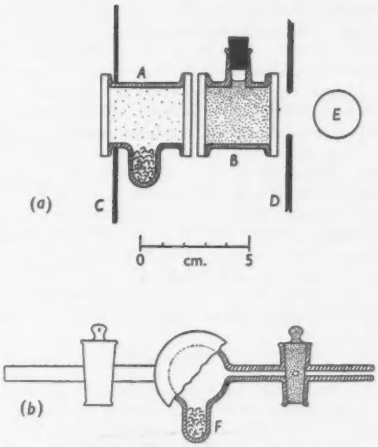

Fig. 1. *a*, Longitudinal section of the chlorine filter (*A*) and liquid filter (*B*) showing their design. *C* and *D* are diaphragms and *E* is the mercury vapour lamp. *b*, Partial transverse section of chlorine filter showing taps used for filling and, *F*, calcium chloride for keeping the gas dry.

followed by a gaseous chlorine filter, was chosen (Bowen, 1946). The filters are of glass with fused quartz windows cemented on. For the liquid filter a resinous cement is used, but for the chlorine filter vaseline is used, a few dabs of Picein wax round the outside holding the assembly firm. The liquid filter, which contains an aqueous solution of $NiSO_4.7H_2O$, 350 g. and $CoSO_4.7H_2O$, 100 g./l., is 30 mm. thick and the gas filter containing chlorine at atmospheric pressure dried over $CaCl_2$ is 35 mm. thick. The cells have a free aperture of 22 mm. and their design is shown in Fig. 1. It seems to be important to have the liquid filter nearer the lamp, as otherwise the chlorine gas will need renewing frequently, whereas if they are arranged as recommended the gas persists indefinitely. A useful check on the efficiency of the filters is given by observing the spectrum of the light transmitted with a Beck ultraviolet spectroscope. The only lines visible should be at 253·7 and 265 mμ., the latter being much less intense. If such an instrument is not available, it is possible to test the light source by printing out a few test spots of adenine on Whatman no. 1 paper. It should be possible to detect 1 μg. of adenine in a 10 μl. spot quite easily, whilst 5 μg. in the same volume should prove almost completely opaque. It is, in fact, possible to detect quantities of materials which are much too small to estimate accurately by means of a spectrophotometer.

The photographic material used is Ilford Reflex Document Paper, no. 50 (similar material is also manufactured by other firms). This material is comparatively cheap, and has the advantage of being extremely contrasting; and it may, if necessary, be handled in subdued artificial light. It is not affected by the yellow light transmitted by the filter system. (Other papers, though faster, have not proved so sensitive to small amounts of absorbing material.) We have not found that the developers used modify the results greatly and either the maker's formula I.D.36 or a process developer (Kodak D153) seems quite satisfactory.

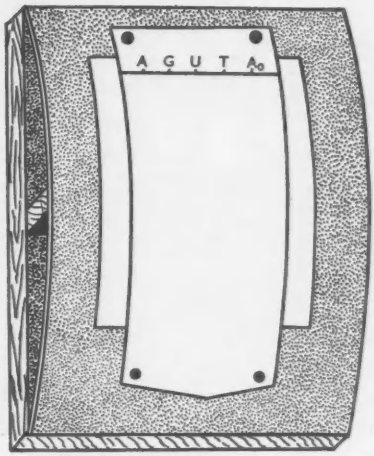

Fig. 2. Printing the chromatogram. The photographic paper is held flat by the filter-paper chromatogram which is pinned over it on the convex surface of the plywood.

Exposure. Under our conditions a suitable exposure is 1·5 min. at a distance of 1·2 m. (which covers an area of about 15 × 40 cm.). For larger areas such as are needed for two-dimensional work, the light source has to be farther away, and the exposure may be estimated approximately by applying the inverse square law. The exposures are not very critical.

For making the prints it would be desirable to have a printing frame of sufficient size with a fused quartz window, but for all practical purposes we find that pinning the chromatogram paper over the photographic paper on a plano-convex surface (a piece of plywood fastened to a board with a small strip of wood under the centre to bow the former out a little (Fig. 2)) is entirely satisfactory.

After development the paper is fixed and washed in the usual way. If it is necessary to dry the paper rapidly it should be bathed after washing in ethanol or acetone and then dabbed with a clean cloth. Dimensional changes in the print appear to be negligible, but if they should be a source of trouble similar emulsions may be obtained coated on waterproof paper, cellulose acetate or glass.

The positions of absorbing substances may be seen on the developed papers as light areas (Fig. 3) and these prints serve as a permanent record of each experiment.

For many purposes the photographic image alone will suffice, but if the spots need to be extracted, their positions may be found by shining a light through the photographic paper and drawing their outlines on the filter paper, which is registered by means of pencil lines or other reference marks made on the latter and which show up on the print. As a general routine we write all the necessary data in pencil on the filter paper so that it is recorded on the print as well. The areas of filter paper corresponding with the spots on the photographic print are cut out and extracted in 5 ml. of water or 0·1 N-HCl for estimation in a Beckman photoelectric spectrophotometer. Areas identical in size are cut from adjacent strips for use as blanks (see Hotchkiss, 1948).

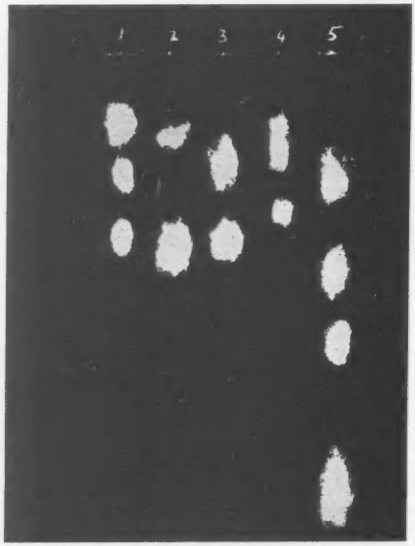

Fig. 3. Print of chromatogram run for 9 hr. in *n*-butanol-formic acid to illustrate points in text. Mixtures were put on at spots 1–5 and each column reads from the top downwards. (1) Uric acid, 6:8-dihydroxypurine and hypoxanthine, and (2) guanine and adenine, both showing effect of OH groups; (3) adenosine and adenine, and (4) cytidine and cytosine, showing effect of ribose; (5) xanthine, 3-methylxanthine, 3:7-dimethylxanthine (theobromine) and 1:3:7-trimethylxanthine (caffeine) showing effect of methylation. (*Note.* To resolve all these substances when present together several solvents would have to be used and the chromatograms run for longer times.)

(*b*) *Chromatographic technique*

All chromatograms were run on Whatman no. 1 paper. For single-dimensional work sheets 13 × 33 cm. were run in glass battery jars. As the R_F values of most of the substances investigated (excluding certain methylated derivatives) were less than 0·5, the lower edge of the paper was cut to a point and the solvent front was run well beyond the end of the paper and a substance of known R_F value, usually adenine, was used as a marker. Two-dimensional chromatograms were run on standard sheets of filter paper in large glass-sided boxes. Solutions were placed on the paper in small areas 6 cm. from the upper edge, either with small glass capillary tubes, or, for quantitative work, with a Burroughs Wellcome Agla syringe. Volumes of 10–20 μl. of solutions containing less than 50 μg. of individual substances were used.

(*c*) *Choice of solvent*

Naturally in this work solvents such as phenol, quinoline, collidine, etc., which absorb heavily in the ultraviolet and are difficult to remove from the paper must be avoided if possible. Several aliphatic alcohols and ketones were tried, but *n*-butanol saturated with water (14% water in butanol (v/v); Hotchkiss, 1948) proved the most useful basic solvent for the separation of the bases and the nucleosides. For a satisfactory differentiation between hydroxy- and amino-purines and pyrimidines (Fig. 3), either ammonia was added to the vapour phase or the solvent was acidified. The systems used most frequently were: (1) a mixture containing 90% *n*-butanol saturated with water and 10% glacial formic acid (v/v); (2) *n*-butanol saturated with water in the presence of an atmosphere containing NH_3; (3) *n*-butanol saturated with water. For two-dimensional chromatograms a combination of (1) and (2) will separate all the substances likely to be encountered in nucleic acid hydrolysates (including xanthine and hypoxanthine, traces of which are usually present).

(*d*) *Materials*

Cytidine and uridine were obtained from Dr J. Davoll and yeast adenylic acid from Dr T. Mann. The methylated xanthines and 6:8-dihydroxypurine were from Prof. D. Keilin, F.R.S. Cytosine was obtained by hydrolysis of cytidine at 145° in 6 N-HCl. Though hydrolysis was practically complete no trace of uracil was found, although we have confirmed the observation of Vischer & Chargaff (1948*b*) that during the acid hydrolysis of nucleic acids some uracil is formed from cytosine. Thymine was obtained from thymus nucleic acid by hydrolysis. The other substances were commercial products.

RESULTS

Table 1 gives the positions on the chromatograms of purines and pyrimidines and some of their derivatives in various solvents. This table is only intended as a guide to the relative positions of these substances, as we find a certain amount of variation from time to time. For instance, particularly in butanol-ammonia systems, the pyrimidines, uracil and cytosine (and their corresponding ribosides) are very sensitive to changes in the composition of the vapour phase and they may change their relative positions. In such chromatograms it is necessary to use adenine and uracil as markers. In the butanol-formic acid system, adenine and hypoxanthine may undergo a similar displacement. When running two-dimensional chromatograms it is essential to use

Table 1. *R_F values of purines, pyrimidines and ribosides in various solvents*

Solvent system* ...	*n*-Butanol 86%:water 14%	*n*-Butanol 86%:water 14%:+NH_3†	*n*-Butanol 77%:water 13%:formic acid 10%	*n*-Butanol 50%:ethanol 15%:water 35%	Amyl alcohol saturated with water	Amyl alcohol saturated with water+NH_3†	Amyl alcohol saturated with water 90%:formic acid 10%
Purines:							
Hypoxanthine	0·26	0·12	0·30	—	0·15	0·02	0·19
Xanthine	0·18	0·05	0·24	—	0·10	0	0·16
6:8-Dihydroxypurine	0·18	0·04	0·24	—	0·07	0	0·11
Uric acid	0·01	0	0·14	—	0	0	0·06
3-Methylxanthine	0·29	0·13	0·32	—	0·19	—	—
1:3-Dimethylxanthine (theophyllin)	0·52	0·22	0·64	—	0·54	0·12	0·55
3:7-Dimethylxanthine (theobromine)	0·42	0·27	0·47	—	0·28	0·15	0·36
3:8-Dimethylxanthine	0·42	0·18	0·48	—	0·34	—	—
1:3:7-Trimethylxanthine (caffeine)	0·63	0·65	0·71	—	0·56	0·50	0·67
Adenine	0·38	0·28	0·33	0·55	0·28	0·16	0·12
Guanine	0·15	0·11	0·13	0·37	0·05	—	0·04
Purine ribosides:							
Adenosine	0·20	0·22	0·12	0·50	0·11	0·09	0·04
Guanosine	0·15	0·03	0·17	0·40	0·02	0	0·04
Pyrimidines:							
Cytosine	0·22	0·24	0·26	0·53	0·09	0·09	0·07
Uracil	0·31	0·19	0·39	0·46	0·22	0·08	0·23
Thymine	0·52	0·35	0·56	—	0·40	—	—
Pyrimidine ribosides:							
Cytidine	0·12	0·11	0·18	0·42	0·02	0·03	0·03
Uridine	0·17	0·08	0·25	0·49	0·05	0·03	0·07

* Composition of solvents is given in terms of volume percentages.
† 5% by vol. of NH_3 solution (sp.gr. 0·880) was added to the solvent mixture in the bottom of the container.

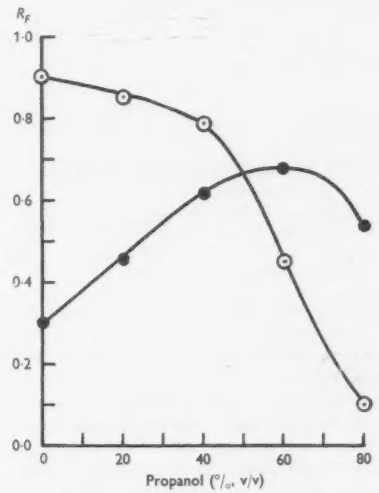

Fig. 4. The variation of R_F values of adenylic acid (open circles) and adenine (solid spots) in solvents of varying water content.

separate markers in each dimension. A print is then taken of the markers after running in each solvent. The markers are, of course, placed on the other end of the top edge of the filter-paper sheet and thus do not interfere.

Solvents which contain little water do not bring about much movement of the phosphorylated derivatives (nucleotides, dinucleotides, etc.) owing to the much greater relative solubility of these substances in water, and consequently they are to be found at the position of the original spot in butanol-water chromatograms. Some separation of these substances may be obtained by the use of solvents based on ternary mixtures such as butanol-ethanol-water and on completely miscible solvents such as propanol-water (Fig. 4).

DISCUSSION

From Table 1 it appears that certain generalizations as to the movement of substances containing certain groupings may be made, and we enumerate them as follows:

(1) Pyrimidines move faster than the corresponding purines. For example, uracil runs faster than xanthine and cytosine faster than guanine

(2) In the presence of ammonia the amino derivatives move much faster than the corresponding hydroxy compounds, but on changing to an acidic solvent this difference is greatly decreased and the relative positions may even be reversed. This is particularly evident in the case of the pyrimidines cytosine and uracil (and their ribosides) in which the reversal takes place at neutrality.

(3) Increase in the number of hydroxyl groups possessed by a substance decreases its rate of movement in all the solvents. This is illustrated by a comparison of the R_F values of hypoxanthine, 6:8-dihydroxypurine and uric acid, and those of adenine and guanine, adenosine and guanosine.

(4) Ribosides move more slowly than the free bases but maintain the same relative positions.

(5) Increasing methylation tends to increase the movement of the substances in all the solvents.

Comparing these results with those of other workers some points of interest may be noted. We find, contrary to Hotchkiss (1948), that, when present in amounts small enough to be completely in solution in the aqueous phase, guanine moves at an appreciable rate in butanol mixtures, and probably corresponds with the 'epiguanine' to which he refers. This is probably due to the fact that his method has too poor a resolution to give a satisfactory picture of the chromatogram as a whole.

Except in acidic solvents, uracil always runs in our chromatograms more slowly than adenine, contrary to the observations of Vischer & Chargaff (1948*a*). These and other minor discrepancies would appear to be due, in part at least, to the different filter paper used by these authors.

SUMMARY

1. A micromethod for the detection and estimation of purines, pyrimidines and related substances is described. It is based upon the detection of spots of these compounds on paper chromatograms by means of a simple contact-printing technique using photographic paper and filtered ultraviolet light, and the sensitivity is such that a few micrograms may be detected.

2. The R_F values of a number of compounds in several solvents are tabulated.

3. Certain generalizations are made correlating chemical structure with the chromatographic behaviour of these substances.

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Studies in Vitamin A

10. VITAMIN A_1 AND RETINENE$_1$ IN RELATION TO PHOTOPIC VISION

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Scotopic (or low intensity) vision is related to the activity of rods, and photopic (or daylight) vision operates by means of cone receptors. When light falls only on rods there is no sensation of colour, and the spectral distribution of sensitivity in scotopic vision can be interpreted in terms of one receptor substance, rhodopsin. For photopic vision, however, the phenomena of colour sensation cannot be explained on the basis of a single absorbing entity with a spectrum corresponding to the photopic sensitivity curve. Nevertheless, the full cone response curve obtained by electrophysiological methods is very similar to the normal photopic sensitivity curve.

One interpretation of the Young-Helmholtz theory is that it implies the existence of three selectively absorbing substances sensitive to different spectral ranges. Many workers have attempted to measure the responses of different receptors to different wavelengths. The usual procedure was first to expose the eye to filtered (or better monochromatic) light in the hope that the receptor affected would be put out of action by photochemical exhaustion. The response of another receptor to

wavelengths of monochromatic light within its own range of sensitivity could then be determined. Prolonged exposure of the eye to one wavelength, however, always produced a reduced response to all wavelengths.

Since 1936, Granit has studied the problem by electrophysiological methods (for review see Granit, 1947). The complicating rod phenomena were eliminated by pretreatment of the eye with light sufficiently intense to bleach all the rhodopsin. After removing the lens and cornea, and applying microelectrodes directly to the retina, Granit was able to pick up responses from individual nerve fibres when monochromatic light fell on the retina, and in that way made fundamental advances in the combined problems of colour sensation and photopic vision. His scotopic action spectra for frog, toad, cat, rabbit, pigeon, guinea pig, fresh-water eel, etc. were very uniform and enabled him to compute the absorption spectrum of rhodopsin. With light-adapted eyes striking results were obtained. Sensitivity curves widely different from the normal photopic sensitivity curve were often recorded, and as a result of many different series of experiments Granit advanced his dominator-modulator theory.

A dominator is a sensory mechanism, whether scotopic or photopic, characterized by a broad sensitivity curve and making available for vision a large range of wavelengths. The differences between the photopic and scotopic dominators account for the Purkinje shift, and the scotopic dominators correspond with rhodopsin or porphyropsin, depending upon whether the eye under study makes use of vitamin A_1 or vitamin A_2. The fact that the photopic dominator response curves are always displaced by some 60–70 mμ. in the direction of longer wavelengths compared with the corresponding scotopic curves whether the eyes contain vitamin A_1 or A_2 or both, suggests that the receptor substances may all be related to the vitamins A. The dominator curves are concerned with the sensation of brightness and are too broad to allow them an important role in colour discrimination.

A modulator, on the other hand, is characterized by a much narrower response curve. The substantial body of data accumulated by Granit shows that the modulator action spectra fall into groups situated in three spectral regions: 440–470, 520–540 and 580–600 mμ. There is an added complication in the existence of a light-sensitive modulator near 500 mμ. The most light-resistant modulator appears to be that near 600 mμ. The narrow action spectra make the modulators peculiarly suitable for colour sensitivity. There is independent evidence that colour mechanisms are not responsible for brightness discrimination (Wright & Granit, 1938).

The outstanding implication of Granit's work, from the present point of view, is that his action spectra provide serviceable 'labels' for hypothetical light-absorbing substances concerned in vision, provided that the relationship between absorption spectra and action spectra is borne in mind (see Ball, Collins, Morton & Stubbs, 1948; Dartnall & Goodeve, 1937; Hecht, 1937; Wald, 1938).

Von Studnitz (1932) bleached isolated retinas and measured the changes in light absorption at different wavelengths, but the evidence he produced for a photosensitive cone pigment is technically questionable. He later (von Studnitz, 1937) extracted from frog retinae an ether-soluble material with $\lambda_{max.}$ 560 mμ.; but when his data are replotted on a scale suitable for comparison with photopic sensitivity it seems that the absorption curve was broader than the 'dominator' curve. For the tortoise (*Testudo graeca*) he recorded $\lambda_{max.}$ for the extract at 560 mμ., although the photopic sensitivity maximum is at 600–610 mμ. Using the snake *Tropidonotus* he obtained extracts with absorption maxima at 468, 560 and 650 mμ. Chase (1938) extracted a water-soluble cone substance (absorption maximum 530 mμ.), and Wald (1938) deduced from differential photodecomposition of chicken retinae an absorption maximum at 575 mμ., which he attributed to a cone pigment, iodopsin. Hosoya, Okita & Akune (1938), using the rod-free retinae of tortoises, claimed to have extracted by means of 2% sodium cholate three water-soluble pigments the absorption spectra of which showed maxima at 460, 570 and 670 mμ. respectively, but the data were not very satisfactory. Hanström (1940) extracted the macular region of monkey retinae and found maxima at 460 and 590 mμ. due to ether-soluble substances.

Bliss (1946*a*, *b*) has confirmed the existence of Wald's iodopsin in chicken retinae and has been able to extract it, although not in a very 'pure' state. The iodopsin was more labile than rhodopsin, and bleaching by light produced retinene in 'large' amount, but there was no detectable change in pH or inorganic phosphate concentration.

That a deficiency of vitamin A impairs scotopic vision is by now familiar, but the relationship of vitamin A to photopic vision is not so clear cut. Inasmuch as there is no real hint of any other key substance in vision, the next step is to find out whether vitamin A is capable of being the only key substance.

Vitamin A and retinene give rise to deep blue solutions with antimony trichloride in chloroform, and the sharpness of the absorption bands, coupled with the transient nature of the blue materials, suggests that under suitable environmental conditions vitamin A or retinene might give rise to 'ionized' or 'halochromic' molecules (see Meunier & Vinet, 1947) resembling the modulators of Granit. When vitamin A or retinene is dissolved in concentrated sulphuric acid or syrupy phosphoric acid at temperatures near 0°, coloured solutions are produced which exhibit well-defined selective absorption with maxima corresponding closely with Granit's maxima. These results are recorded and discussed below.

EXPERIMENTAL

The materials used were crystalline samples of vitamin A alcohol and retinene$_1$, the ionizing media being H_2SO_4 (sp.gr. 1·84) and H_3PO_4 (sp.gr. 1·75). The Beckman spectrophotometer was used for the quantitative measurement of absorption spectra, the Hilger-Nutting visual instrument for some qualitative determinations.

RESULTS

Vitamin A and sulphuric acid

A few minute crystals of vitamin A were added to about 20 ml. of concentrated sulphuric acid and the mixture was well shaken. The vitamin dissolved easily, and a transient purple colour was immediately formed ($\lambda_{max.}$ 620 mμ.). The blue component of the colour very quickly faded, and the solution became bright red with absorption maxima at 465, 520 and 580 mμ. By means of the Hilger-Nutting instrument the diminution in intensity and disappearance of the 620 mμ. band with concomitant appearance and increasing intensity of the 580 mμ. band could be observed, showing clearly an interrelationship between the substances responsible for these absorption maxima. The 520 mμ. band was also unstable and disappeared fairly rapidly at room temperature even in the dark. If the sulphuric acid solution was kept in the solid state at $-78°$ in the dark, all three bands persisted for several days. Under such conditions, i.e. dissolution of vitamin A in very cold sulphuric acid, an absorption band was found at 560 mμ. instead of 580 mμ.

When, immediately after mixing, the sulphuric acid solution of vitamin A was agitated with light petroleum, *cyclo*hexane or ether, coloured materials did not pass into the organic solvent and ultraviolet absorption measurements on the extracts showed only the presence of unchanged vitamin A. When chloroform was used as the extracting solvent a cloudy solution was obtained which developed a pink colour on standing. The absorption spectrum of this chloroform extract (Fig. 1) showed bands at 460 and 555 mμ., due to separate chemical entities, since the absorption bands disappeared at different rates under the influence of heat or light.

A dilute dispersion of concentrated sulphuric acid in chloroform was prepared by shaking a mixture of chloroform and sulphuric acid vigorously for 10 min. and allowing to settle. The chloroform solution was slightly cloudy and an intense bluish red colour was immediately obtained on addition of vitamin A. The absorption spectrum of this coloured solution (Fig. 2) was complicated and exhibited maxima at 470 and 570 mμ. On standing for 15–25 min. in light or the dark the spectrum was resolved into a series of absorption bands.

A solution of vitamin A in ethanol gave an intense blue colour with sulphuric acid ($\lambda_{max.}$ 620 mμ.), which changed rapidly to a bright red ($\lambda_{max.}$ 560 mμ.). This red colour also disappeared very quickly leaving a dirty brown solution which showed no selective absorption in the visible or ultraviolet.

Attempts were made to fractionate the coloured products of the sulphuric acid reaction, but without much success. Dilution with water at 0°, followed by neutralization with sodium carbonate or barium carbonate produced colourless solutions exhibiting no selective absorption in the ultraviolet. The best results were obtained by mixing solutions of vitamin A in ethanol with sulphuric acid at temperatures just high enough to keep the acid liquid.

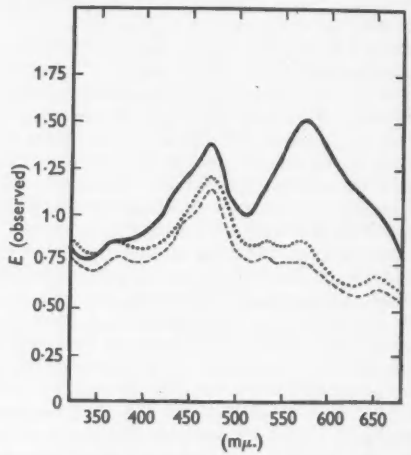


Fig. 1. Absorption spectrum of chloroform extract of a solution of vitamin A in sulphuric acid. ——, measured immediately; - - - - -, after 24 hr. in the dark; . . . . ., after 24 hr. in diffused daylight.

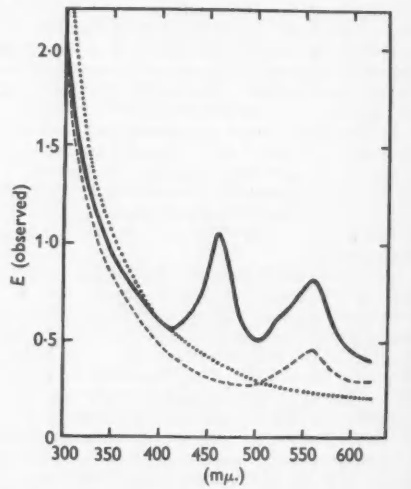


Fig. 2. Absorption spectrum of vitamin A in a chloroform dispersion of sulphuric acid. ——, measured immediately; - - - - -, after 15 min. of diffused daylight; . . . . ., after 25 min. in the dark.

These mixtures were diluted with ice and water, treated with anhydrous sodium carbonate until effervescence ceased, and then extracted with chloroform. The chloroform solutions obtained in this manner were bluish green in colour, the absorption spectra showing maxima at 380 and 465 mμ. The coloured products were stable at −80° in the dark, but faded rapidly on exposure to light or warming to room temperature.

Vitamin A and phosphoric acid

A sample of vitamin A ($E^{1\%}_{1\,cm.}$ 980 at 326 mμ. in *cyclo*hexane) dissolved in a little ethanol, was stirred into phosphoric acid. A blue solution ($\lambda_{max.}$ 620 mμ.) was obtained at first, but rapidly became red with absorption maxima at 480, 540 and 600 mμ. (Fig. 3).

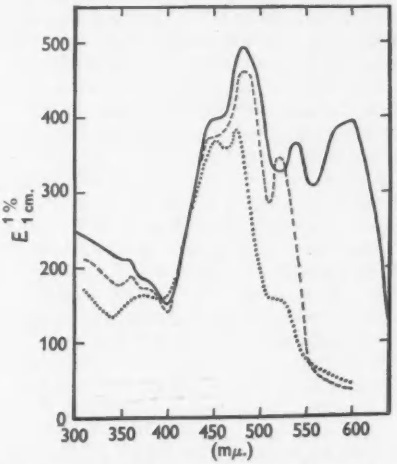


Fig. 3. Absorption spectrum of vitamin A in phosphoric acid. ——, measured immediately; - - - - -, after 5 hr. in the dark; . . . . ., after 5 hr. in diffused daylight.

The 600 mμ. maximum disappeared quickly at room temperature in the dark, but the 480 mμ. band persisted under these conditions. However, the 480 mμ. band decreased in intensity on exposing the solution to light, and after 5 hr. absorption bands of approximately equal intensity were found at 450 and 470 mμ. After 5 hr. in the dark, the 540 mμ. maximum was displaced to 520 mμ., while a similar sample exposed to the light showed only an inflexion at 520 mμ.

Retinene and sulphuric acid

If retinene is dissolved in chloroform and the solution shaken with sulphuric acid, the acid layer becomes bright red in colour and the chloroform deep blue ($\lambda_{max.}$ 664 mμ.). Using light petroleum as the solvent for retinene, no colour develops in the petroleum phase, although the acid layer again becomes red. Treatment of solid retinene with sulphuric acid gave a purplish red solution, the blue component of which disappeared rapidly, leaving a red colour. A complex absorption spectrum was observed with narrow absorption bands at 380, 440, 460, 520 and 560 mμ. (Fig. 4). These absorption maxima disappeared rapidly at room temperature, but persisted well at −80°. After dilution with ice and water, the acid was neutralized with anhydrous sodium carbonate and the solution shaken with chloroform. A colourless chloroform extract was obtained, the spectrum showing no selective absorption in the ultraviolet region. When this chloroform solution was treated with the antimony trichloride reagent a red coloration was obtained,

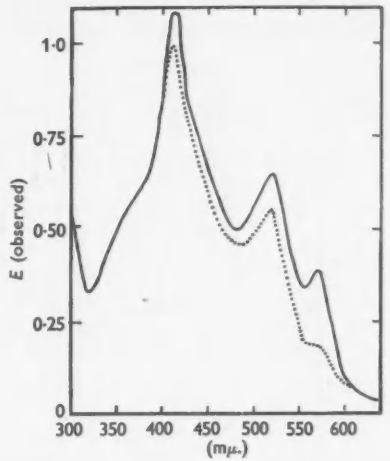


Fig. 4. Absorption spectrum of retinene in sulphuric acid. ——, measured immediately; . . . . ., after 5 min. in diffused daylight.

the absorption spectrum showing maxima at 495, 525 and 590 mμ.

When a solution of retinene in ethanol was stirred into sulphuric acid, the temperature being just above the melting point of the acid, an intense blue colour was obtained ($\lambda_{max.}$ 664 mμ.). This rapidly changed to ruby-red, absorption maxima being observed at 420, 520 and 570 mμ. The solution was diluted, neutralized and extracted as described above; the chloroform extract showed no selective absorption in the visible and ultraviolet regions of the spectrum. With the antimony trichloride reagent a red solution was obtained, the absorption spectrum of which showed bands at 495, 525 and 590 mμ. as above. In the original acid solution it was observed that exposure to light reduced the intensity of each

absorption band, the 570 mμ. maximum diminishing more rapidly than the others. After 5 min. in diffuse daylight the 570 mμ. band had disappeared, whereas the other bands were still quite intense.

With sulphuric acid dispersed in chloroform retinene gave a blue solution ($\lambda_{max.}$ 664 mμ.) which soon became red. The absorption spectrum then exhibited maxima at 390, 515 and 560 mμ., all three bands being reduced in intensity by exposure to light or standing in the dark at room temperature.

Retinene and phosphoric acid

Solid retinene dissolved readily in syrupy phosphoric acid to produce a red solution, a transient blue colour being formed first. The coloured substances were markedly unstable unless the solution was kept at −80°, but increased stability was achieved by dissolving the retinene in a little ethanol

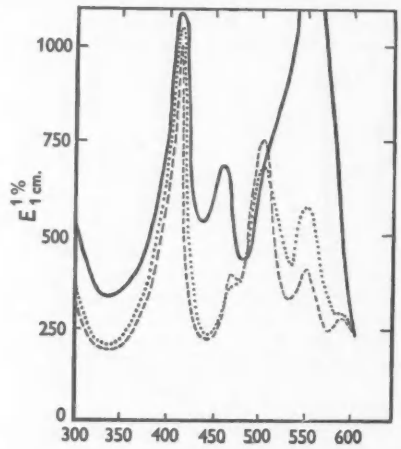


Fig. 5. Absorption spectrum of retinene in phosphoric acid. ——, measured immediately; ....., after 2 hr. in the dark at 0°. - - - - -, after 2 hr. in diffused daylight at room temperature.

before adding the acid. The blue coloration was then recognized as due to a substance with $\lambda_{max.}$ at 664 mμ., and further bands were found at 410, 460 and 560 mμ. in the absorption spectrum measured shortly after mixing (Fig. 5). A pronounced inflexion was observed at 520 mμ. After the solution had been left in the dark at 0° for 2 hr. the absorption spectrum was remeasured. The 560 mμ. maximum had decreased in intensity by more than half, the 460 mμ. maximum had disappeared and the 520 mμ. inflexion had become a sharp maximum at 500 mμ. The 410 mμ. maximum had remained unchanged during this time, and new absorption bands of low intensity were apparently responsible for the pronounced inflexions at 470 and 585 mμ. These changes are recorded in Fig. 5, together with the results obtained when a similar solution was kept at room temperature in diffuse daylight for 2 hr.

A quantitative determination of the intensity of the 560 mμ. maximum was rapidly carried out, the starting material being retinene of $E^{1\%}_{1\,cm.}$ 1400 at 383 mμ. in ethanol. Although the band was decreasing rapidly during the measurement, an $E^{1\%}_{1\,cm.}$ value of 2480 was obtained at 560 mμ. It dropped to 1884 in 3 min., and in 2 hr. there was no maximum at that wavelength. Many similar measurements were made, but it was difficult to distinguish sharply between thermal and photochemical processes. It seems, however, probable that the substances responsible for the 410 and 500 mμ. bands are stable to light even at room temperature, while the 560 and 460 mμ. materials are labile to both heat and light. Examination of the different series of results obtained indicated that the absorption bands at 410–460 mμ., 560 and 664 mμ. are due to separate chemical entities, because they did not appear and disappear together. It is probable, however, that the material responsible for the 664 mμ. band is the precursor of the other substances, since it was always produced first and as the 664 mμ. maximum diminished so the other absorption bands became more prominent.

When solutions of retinene in ethanol are mixed with phosphoric acid, a series of colour changes occurs. The solutions are blue immediately after mixing, and pass through violet and red stages before becoming brown. Attempts made to extract fractions of the coloured materials at different stages by shaking with common organic solvents met with little success. The coloured substances invariably remained in the acid phase. Dilution with ice and water before extraction produced a yellow chloroform extract, the absorption spectrum being flat and featureless, except for a maximum of low intensity at 300 mμ. When this chloroform solution was treated with the $SbCl_3$ reagent a red solution was obtained, the absorption spectrum of which showed intense bands at 475 and 520 mμ., and weak bands at 505 and 535 mμ.

Additional observations. The use of concentrated hydrochloric acid gave results similar to those obtained with phosphoric and sulphuric acids, though in this case the absorption spectra were not measured in detail. However, the same colour changes were noted, i.e. transient blue, passing through intense red to dirty brown, the sequence of changes being very much more rapid with hydrochloric acid than with either of the other acids.

Attempts to fractionate the different materials by chromatography failed, although both kieselguhr and alumina columns adsorbed the coloured products from the chloroform-acid-vitamin A reaction mixture. The substances remained adsorbed in a

narrow zone at the top, irrespective of which organic solvent was used for development. It was found that the colour reactions of vitamin A and retinene with sulphuric acid were inhibited by dilution of the acid with water. In 50 and 80 % (v/v) concentrations, sulphuric acid gives no coloration with vitamin A or retinene.

Table 1. *Comparison of the action spectra of Granit's modulators and the absorption spectra of vitamin A and retinene dissolved in strongly acidic solvents*

(Wavelengths of maximum absorption, $\lambda_{max.}$, in mμ.)

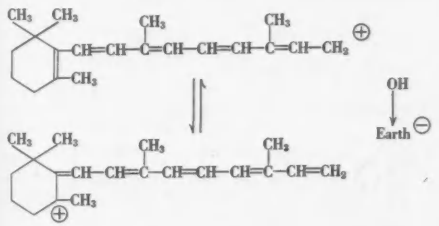
Rhodopsin	—	500	—	—	—	—
Iodopsin	—	—	—	560	—	—
Dominators	—	500	—	560	—	—
Modulators	450–465	500	520–530	—	580–610	—
Vitamin A in conc. H_2SO_4	465	—	520–530	—	590	620
Vitamin A in conc. H_3PO_4	440–480	—	520	—	—	620
Retinene in conc. H_2SO_4	440–460	—	520	560	—	664
Retinene in conc. H_3PO_4	470	500	—	550	590	664

DISCUSSION

The absorption spectra observed when vitamin A or retinene is dissolved in concentrated sulphuric acid or syrupy phosphoric acid bear a striking resemblance to the action spectra of Granit's modulators (see Granit, 1947). This similarity is obvious from the summary of both sets of results given in Table 1.

Granit's modulator maxima fall roughly into three spectral ranges, and the absorption maxima obtained by the mineral acid reactions also fall into similar groups. Some additions to Granit's maxima must be noted, e.g. the 560 and 550 mμ. bands obtained from retinene.

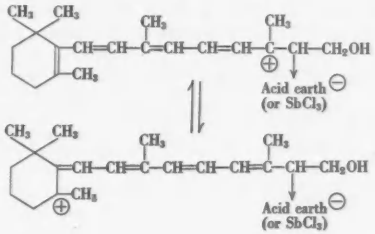
There can be little doubt that the absorption bands obtained are characteristic of 'ionized' or 'halochromic' molecules, as has been postulated by Meunier (1942) for the blue colour obtained when vitamin A is treated with the antimony trichloride reagent. Vitamin A gives a similar blue colour when



adsorbed on certain acid earths, e.g. montmorillonite, and Meunier (1942) suggested that the mechanism was similar to that of the antimony trichloride reaction. These acid earths (or antimony trichloride) possess incomplete electronic octets and are able to accept unshared electrons from the vitamin A molecule, which undergoes polarization and forms positively charged strongly resonating structures.

As a first possibility, Meunier (1942) suggests that the hydroxyl group of vitamin A attaches itself to the acid earth leaving a positively charged carbonium ion. With this type of structure the positive charge would resonate through a system of five conjugated double bonds giving rise to the 620 mμ. maximum.

As a second mechanism Meunier postulated a polarization of the double bond nearest to the primary alcoholic grouping and resonance between the following limiting forms:



With this type of structure the positive charge would resonate through a system of four conjugated double bonds giving rise to the 590 mμ. maximum. Meunier & Vinet (1947), carrying the procedure still further, suggest that resonance of a positive charge through two and three conjugated double bonds would give rise to maxima at 500 and 540 mμ. respectively.

The absorption bands obtained with inorganic reagents are apparently narrower than those implied by Granit's action spectra; this difficulty will be discussed in a later paper.

The photopic receptor substances, presumably conjugated proteins, must be present at the cone surfaces; they must be relatively thermostable as otherwise there would be a spontaneous sensation of light (de Vries, 1948), and when detached from the protein moiety, the halochromic or ionized pros-

thetic grouping derived from vitamin A or retinene would yield no materials not known to be present in the eye.

CONCLUSIONS

The modulator analogues which have been obtained fulfil nearly all the requirements of the colour receptors whose presence is implied by Granit's work, e.g. during fading processes, the maxima fall at different rates, suggesting the presence, not of one product with several bands, but of several products with one maximum to each. The conditions under which these modulator analogues have been produced are obviously unphysiological.

If the problem posed by Granit's modulators is put in the form of a query whether or not the vitamin A-retinene oxidation-reduction system could possibly account for the phenomena observed, the answer is clearly in the affirmative. Which catalysts, old or new, must be invoked before the modulator analogues can be obtained under less obviously unphysiological conditions is a matter for further research.

SUMMARY

1. The absorption spectra of vitamin A and of retinene$_1$ in concentrated sulphuric, phosphoric and hydrochloric acids have been studied. Sharp absorption bands characteristic of unstable ionized molecules were obtained.

2. The results show that vitamin A and retinene$_1$ can give rise *in vitro* to materials simulating the photopic modulators of Granit.

3. The hypothesis that the system vitamin A-retinene$_1$ is important in photopic as well as scotopic vision is strengthened.

We are indebted to the Medical Research Council and the Ministry of Food for financial assistance, and to Dr A. L. Stubbs and Dr F. D. Collins for help in the course of the work.

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Studies in Vitamin A

11. REACTIONS OF RETINENE$_1$ WITH AMINO COMPOUNDS

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Rhodopsin or visual purple is a conjugated protein obtained from dark-adapted retinae of many species. The prosthetic group is responsible for the colour ($\lambda_{max.}$ 500 mμ.) and photosensitivity, and is derived from vitamin A or its aldehyde retinene$_1$. No explanation has so far been advanced to account satisfactorily for the displacement of $\lambda_{max.}$ from either 328 mμ. (vitamin A) or 370–390 mμ. (retinene$_1$) to 500 mμ.

The decomposition product of rhodopsin known as indicator yellow shows $\lambda_{max.}$ 440 mμ. in acid solution and 365 mμ. in alkaline solution. Neither the pH sensitivity nor the 440 mμ. maximum has been properly accounted for.

The interaction of purified retinene$_1$ with amino compounds throws some light on these problems. A preliminary account of the work (Ball, Collins, Morton & Stubbs, 1948) has appeared, and the present paper carries the study a stage further.

EXPERIMENTAL

General procedure (*a*). A solution of crystalline retinene$_1$ in ethanol (about 10^{-6} M) was prepared so that the optical density (*E*) at 380–390 mμ. was 1·5–2·5, for a 1 cm. layer. To the solution (2 ml.) was added an aqueous solution of amino compound (2 ml.). In some cases the latter solution was nearly saturated, and in all cases the concentration exceeded 10^{-3} M. To the mixture, 0·1 N aqueous NaOH (2 ml.) was added, and the final mixture was left to stand at room temperature for 15–30 min. The absorption spectrum of the solution was then measured. Next, conc. HCl (1 drop) was added and the absorption spectrum redetermined, using a compensating cell containing water. The Beckman spectrophotometer was used throughout. In general, the alkaline solutions showed $\lambda_{max.}$ 360–370 mμ. and the acidified solutions $\lambda_{max.}$ 435–460 mμ.

General procedure (*b*). A number of amino compounds in which the NH_2 group is attached directly to the benzene ring produce a reddish colour with retinene$_1$ in the presence of acid. Pretreatment with alkali is not necessary, and $\lambda_{max.}$ appears at 490–535 mμ. depending on the nature of the amino compound.

Conditions of interaction of retinene$_1$ and amino compounds. The effect of varying the molecular ratio amino compound/retinene$_1$ has been investigated in the case of β-alanine.

RESULTS

A variety of compounds containing amino groups has been tried by procedure (*a*). Urea, formamide and dimethylamine significantly failed to shift the

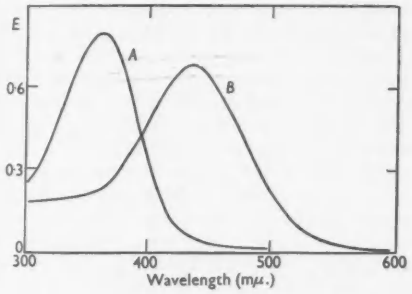


Fig. 1. Absorption spectrum of retinene$_1$ plus methylamine (in excess). *A*, after standing (10 min.) in alkaline medium; *B*, subsequently acidified.

retinene$_1$ absorption spectrum. Simple amines and aliphatic amino-acids, as well as tyrosine and tryptophan, reacted slowly with retinene$_1$ in alkaline solution giving a displacement of $\lambda_{max.}$ from 380–390 to 360–370 mμ. On acidification a new band $\lambda_{max.}$ 435–460 mμ. appeared (Figs. 1 and 2).

The effect lacks specificity, since a variety of proteins show it, and the only difficulty is that instead of an orange yellow solution, an orange precipitate is sometimes formed (Table 1).

Aniline and various compounds related to it produce on the other hand a colour in acid solution immediately on mixing, no treatment with alkali being necessary. (Fig. 3, aniline, Fig. 4, *p-amino*-benzoic acid.) This colour is due to a chromophore with $\lambda_{max.}$ 490–535 mμ. (Table 2).

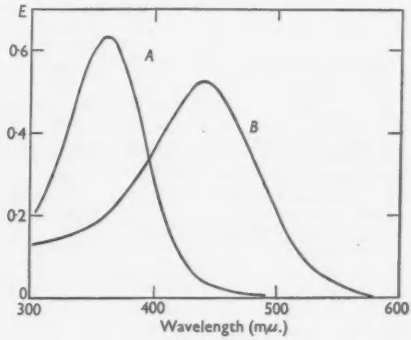


Fig. 2. Absorption spectrum of retinene$_1$ plus β-alanine (in excess). *A*, after standing (10 min.) in alkaline medium; *B*, after subsequent acidification. Retinene concentration $1·3 \times 10^{-6}$ M; 1 cm. cell.

Table 1. *Wavelengths of maximum absorption of mixtures of retinene$_1$ and amino compounds in aqueous ethanol*

(The mixtures were first made alkaline and left to stand and then acidified. The amino compound was in considerable excess over retinene$_1$.)

Amino compound	$\lambda_{max.}$ in alkali (mμ.)	$\lambda_{max.}$ in acid (mμ.)
Methylamine	360–365	435
Dimethylamine	385–390	385–390 (no change)
Benzylamine	365	445
Urea	385–300	385–390 (no change)
Formamide	385–390	385–390 (no change)
Glycine	372	440–445
β-Alanine	365	440
Serine	370	435–440
Isoleucine	370	450–455
Tyrosine	365	445
Tryptophan	360–365	445
Glutamic acid	375	435–440
Lysine	365	440–445
Arginine	365–370	460
Egg albumin	370	450
Peptone	365	440
Edestin	380	455
Trypsin	Solution not clear	440–450
Gelatin	360	440
Casein	360	Orange yellow precipitate
Zein	360–365	Orange yellow precipitate

Table 2. *Wavelengths of maximum absorption of mixtures of retinene$_1$ and amino compounds, which result in visible colour in acid solutions without preliminary treatment with alkali.*

Amino compound	$\lambda_{max.}$ in acid solution (mμ.)
Aniline	490–500
Methylaniline	490
Benzylaniline	490
Dimethylaniline	No colour produced
1-Naphthylamine	500
2-Naphthylamine	515
p-Toluidine	500
o-Aminophenol	505
p-Aminobenzoic acid	530
Anthranilic acid	520
p-Aminobenzenesulphonamide	520
Diphenylamine	535

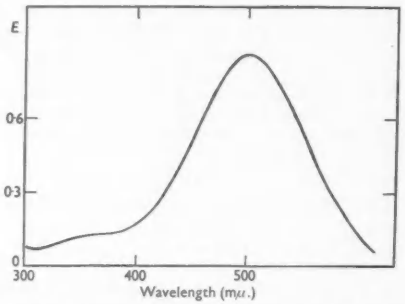


Fig. 3. Absorption spectrum of retinene$_1$ plus aniline (in excess) in acid solution; retinene concentration $1{\cdot}58 \times 10^{-5}$ M; 1 cm. cell.

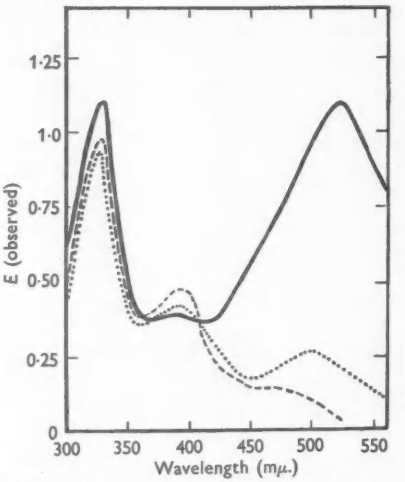


Fig. 4. Absorption spectrum of retinene$_1$ plus *p*-aminobenzoic acid, acidified; ——, solution examined at once, ... , after 6 hr. exposure to diffused daylight, - - - - -, after 10 hr. exposure to daylight.

In procedure (*a*) it is necessary to have the amino compound in excess over the retinene$_1$ if the change in $\lambda_{max.}$ from 380–390 to 360–370 mμ. in alkali is to be effected quickly (e.g. in 10 min.). The molecular ratio of, for example, β-alanine/retinene needs to be about 200, but a smaller excess will show the displacement in 48 hr. (Table 3). Even in quite alkaline media (0·033 M-NaOH) there is no shift in $\lambda_{max.}$ when β-alanine and retinene$_1$ are mixed in equimolecular proportions. With excess of β-alanine the reaction occurs readily in 0·033 M-NaOH, but very slowly in neutral solution (measured pH 6·98), a small shift only being obtained after 48 hr.

Table 3. *Interaction of retinene$_1$ and β-alanine in* 0·033 M-NaOH *and ethanol*

(The mixture was left to stand and then acidified with one drop conc. HCl; retinene, $1{\cdot}3 \times 10^{-5}$ M; β-alanine in excess.)

Molar ratio β-alanine/retinene	Alkaline solution $\lambda_{max.}$ (mμ.)	Acidified solution $\lambda_{max.}$ (mμ.)	Time, NaOH solution was left to stand
0·5	385	385	48 hr.
1	375	385	48 hr.
30	370	395	48 hr.
60	390	395	10 min.
60	365	407	48 hr.
120	370	410–415	10 min.
180	370	425	10 min.
240	365	430	10 min.
480	365	440	10 min.
600	365	440	10 min.

Retinene$_1$ (33 mg.) and β-alanine (5 mg.) were dissolved in ethanol (2 ml.), 0·1 N ethanolic NaOH (2 ml.) added, the mixture allowed to stand for several hours in the dark and then acidified with concentrated hydrochloric acid. The solution became dark red ($\lambda_{max.}$ 440–450 mμ., using a small portion diluted with ethanol). If one drop of the red solution was diluted with water, the colour disappeared and $\lambda_{max.}$ occurred at about 390 mμ. The solute obtained by removal of solvent *in vacuo* from the red solution was insoluble in light petroleum, but soluble in chloroform ($\lambda_{max.}$ 465 mμ.). The red solution showed no spectroscopic evidence of free retinene in appreciable proportion, although the retinene$_1$ and β-alanine were used in the molecular ratio 2 : 1.

Those amino compounds which result in $\lambda_{max.}$ of about 500 mμ. permit an approximately quantitative study of colour intensity (E at 490–500 mμ.) in relation to concentration of reactants and combining proportions. This is possible for the 500 mμ. chromogen because retinene$_1$ has no appreciable absorption at that wavelength, whereas the overlapping of the 440 mμ. absorption and retinene$_1$ absorption is a serious complication. Benzylaniline, which gives $\lambda_{max.}$ 490 mμ. although it has only one available hydrogen, was used for the stoicheiometric test. Table 4 illustrates the results. The assumption

Table 4. *Interaction of retinene$_1$ and benzylaniline in acid solution*

$$K = \frac{[\text{resultant}]}{[\text{retinene}]\,[\text{amine}]}$$

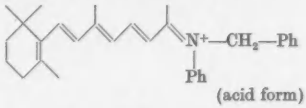
Retinene concentration (M × 10^{-5})	Benzylaniline concentration (M × 10^{-5})	E490mμ. (corr.)	K
1·59	1·59	0·139	$1·85 \times 10^4$
1·59	3·18	0·246	$1·95 \times 10^4$
1·59	7·95	0·392	$1·68 \times 10^4$
1·59	15·9	0·516	$1·69 \times 10^4$
1·59	Large excess	0·723	—

that one molecule of retinene reacts with two molecules of benzylaniline does not lead to an equilibrium constant, but a reasonably good constant is obtained on the basis of one molecule of retinene reacting with one molecule of benzylaniline.

DISCUSSION

To some extent the large excess of amino compounds needed in these experiments reflects the fact that very high dilution is needed for the spectrophotometry. The evidence, however, points to the interaction of two molecules of retinene$_1$ with one molecule of methylamine, glycine, etc., since one molecule of retinene$_1$ with one molecule of aliphatic amine could scarcely give the indicator yellow type of spectrum. This implies that conjugated proteins resembling and including indicator yellow itself possess two retinene$_1$ molecules attached to the same amino nitrogen atom. The molecular extinction coefficients of the methylamine derivative are about 53,000 and 45,600 in alkaline and acid media respectively (calculated/mole of retinene$_1$). The β-alanine derivative shows 'ϵ' values of about 49,000 and 40,000. As the ϵ values for vitamin A and retinene$_1$ are respectively 50,000 and about 40,000, the chromophoric change is confined to a displacement of wavelength.

The structural possibilities for the interaction of retinene$_1$ and *N*-benzylaniline are limited and the structure shown here is the most plausible one. There



(acid form)

are serious objections against the assumption that the results of Table 1 suggest that rhodopsin contains a nitrogen atom attached both to an aromatic ring and a retinene type of molecule—especially since that would imply a different linkage in indicator yellow from that in rhodopsin. Indeed it is not necessarily true that rhodopsin is a retinene, rather than a vitamin A derivative, although indicator yellow is certainly derived structurally from retinene$_1$. These, and other points concerning indicator yellow obtained from retinae, will be discussed in a later paper.

SUMMARY

1. Retinene$_1$, left to stand for a short time in alkaline solution with excess of many amino compounds and proteins, exhibits the absorption spectrum of alkaline indicator yellow, and on acidification shows that of acid indicator yellow.

2. Retinene$_1$, treated in acid solution with aniline and similar compounds, gives a red solution with $\lambda_{max.}$ about 500 mμ. This shows that retinene can give rise to a rhodopsin-like chromophore although in a different way from rhodopsin.

We are indebted to the Medical Research Council and the Ministry of Food for financial assistance. One of us (F.D.C.) is the holder of a New Zealand National Research Scholarship.

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Studies in Vitamin A

12. WHALE-LIVER OIL ANALYSIS: PREPARATION OF KITOL ESTERS

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Whale-liver oil is valued for its vitamin A content, but this has so far been difficult to determine. Indeed, there has been no satisfactory alternative to the biological assay, which is itself of limited utility, if only because the fiducial limits of error ($P = 0{\cdot}95$), using eighty rats, may often be no better than 70–140 % of the estimated potency. Whale-liver oil is, moreover, a very variable material. The crude oil, obtained by processing livers at sea or in Antarctic stations, usually contains phospholipins, water, free fatty acids and vitamin A decomposition products, as well as other impurities. In addition there is kitol, a substance not known to occur in comparable quantity in any other fat. The refined whale-liver oils of commerce also vary considerably. Quite apart from the fairly wide range of vitamin A content in crude oils, the refined oils are obtained in different ways and the resulting products are better described as concentrates. In some, the vitamin A may exist mainly in ester form, and in others it may be predominantly present as the free alcohol. The concentrates are often relatively free from oxidation products derived from vitamin A, but they are rarely, if ever, free from kitol. Anhydrovitamin A (almost certainly an artifact) is also present to a variable extent in whale-liver oil samples.

Some decomposition of vitamin is perhaps unavoidable as a result of the difficulties inherent in working up in factory ships large amounts of liver containing only a low percentage of fat. Opinions have differed as to whether kitol is or is not present in the liver of the living animal. In any case the presence of anhydrovitamin A, of oxidation products and of kitol, gives rise to serious difficulties when the ultraviolet absorption or the antimony trichloride colour test is used to determine vitamin A in whale-liver oils or concentrates. Among the interfering substances, kitol is specially interesting because its biogenesis and possible function await elucidation.

Edisbury, Morgan & Morton (1935) found that, compared with fish-liver oils, whale-liver oils and concentrates exhibited anomalous ultraviolet absorption and abnormal colour tests, and Edisbury, Morton, Pritchard & Wilkinson (1937) described a fractionation of unsaponifiable extracts by partitioning between light petroleum and 83 % (v/v) aqueous ethanol. The fraction soluble in 83 % ethanol contained nearly all the vitamin A, whilst the light petroleum-soluble fraction showed an ultraviolet absorption curve with a maximum at 285–290 mμ. and gave a purple rather than a blue colour with the antimony trichloride reagent ($\lambda\lambda_{max.}$ 594 and 496 mμ.). The $E^{1\%}_{1\,cm.}$ (285 mμ.) value was raised to 240 (which would to-day be regarded as indicating 30–35 % kitol), and the biological assay indicated about 17,900 i.u./g., corresponding possibly to about 0·5 % of residual vitamin A or a very low intrinsic potency for the new substance.

Embree & Shantz (1943) subjected to molecular distillation a de-sterolated whale-liver oil concentrate dissolved in a maize-oil fraction and eliminated vitamin A at 150°. The residue yielded a non-saponifiable fraction, which, after chromatography on alumina, yielded a resin exhibiting $\lambda_{max.}$ 290 mμ., $E^{1\%}_{1\,cm.}$ 580, and in the colour test showing maxima at 428, 505 and 580 mμ. They named the main constituent kitol. When the material was again subjected to molecular distillation elimination began at 175° and became maximal at 225°. The product was, however, less pure than before, because the kitol had decomposed, yielding a certain amount of vitamin A.

Embree & Shantz (1943) reported the following composition and characteristic properties of kitol: Mol. wt. 575. Formula, $C_{40}H_{58}(OH)_2$. Yield of vitamin A by thermal decomposition: 1 mol. vitamin/mol. kitol. $[\alpha]^{25°}_{5461} - 1{\cdot}35$. $\lambda_{max.}$ 286 mμ., $E^{1\%}_{1\,cm.}$ 580.

Baxter, Clough, Kascher & Robeson (1947) obtained a similar product with $E^{1\%}_{1\,cm.}$ (290 mμ.) 586, and from it obtained kitol crystals (m.p. 88–90°) after first obtaining a crystalline di-*p*-phenylazobenzoate. The pure kitol gave $E^{1\%}_{1\,cm.}$ (290 mμ.) 707 and the formula $C_{40}H_{58}(OH_2)$ was confirmed. Geometric isomerism complicated the work on kitol esters. It is thus clear that kitol is a di(vitamin A), but its structure has not been elucidated and it cannot be 'depolymerized' by any method short of pyrogenic decomposition.

The present paper is concerned with (*a*) gaining further information about kitol, in particular whether it is or is not an artifact, and (*b*) the solution of the problem of analysing whale-liver oils for

vitamin A. The background of (*b*) is discussed later (p. 314).

Hickman (1943) has suggested that kitol is a detoxication product in the sense that an 'excessive' accumulation of vitamin A is prevented when the inactive dimeride is formed in the whale liver. This is a legitimate enough speculation, but even its narrower implications have yet to be studied. Thus, the kitol/vitamin A ratio might perhaps vary with the potency of the oil and perhaps with the age of the whale. On the other hand, if kitol were found to be a normal constituent of whale blood and milk the suggestion would need to be reconsidered.

EXPERIMENTAL

Whale milk *Kitol*

Only one sample of milk, and that from a fin whale, has reached us. It contained 16% fat (0·7% of unsaponifiable matter), approx. 380 i.u. vitamin A/100 g. milk and showed ultraviolet absorption consistent with the presence of some kitol (Fig. 1). Examination for kitol of milks from sperm whales and blue whales is desirable, but material is not easily obtained.

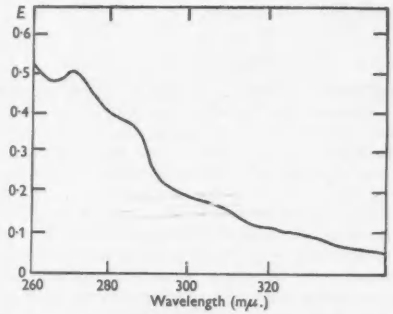

Fig. 1. Ultraviolet absorption spectrum of fin-whale milk lipid, 1·75% in *cyclo*hexane, 1 cm.

Whale-liver oil

The crude commercial oil is usually very dark and highly viscous. Our early attempts to prepare pure kitol esters were hindered by the presence of free fatty acids and phospholipins in the material subjected to chromatographic adsorption. Much better results were obtained with acetone-soluble fractions, particularly those obtained in the laboratory from preserved liver. The ease with which phospholipins dissolve in light petroleum is a disadvantage, because they tend to accompany kitol esters in chromatography. The following section illustrates the difficulty.

Phospholipins of whale-liver oil. Crude sperm-whale-liver oil (2·212 g.) was extracted many times with acetone, and the insoluble residue (0·766 g.) was dried in a current of CO_2. The residue, dissolved in light petroleum, was poured on a column (30 × 1 cm.) of full-strength alumina. The chromatogram was developed with light petroleum. The eluate contained 0·41 g. of material giving no colour with $SbCl_3$ reagent and only weak general absorption in the ultraviolet with a faint inflexion at 275 mμ.

Further development with light petroleum-ether mixture (4 : 1) gave 0·097 g. of a pale yellow oil, $\lambda_{max.}$ 290 mμ., $E^{1\%}_{1\,cm.}$ 121, and ether-ethanol mixtures gave 0·153 g. of pale oil, $\lambda_{max.}$ 295 mμ., $E^{1\%}_{1\,cm.}$ 157. Ether extracted most of the residual material. There seems to be in whale-liver oil a substance of which kitol is a component and possessing an $E^{1\%}_{1\,cm.}$ value near 160.

By dissolving crude sperm-whale-liver oil in light petroleum and adding acetone, a phospholipin fraction is thrown out of solution. The insoluble material may be redissolved in petroleum and reprecipitated by means of acetone. If this is repeated several times the material adsorbing selectively at 290 mμ. can be removed. The residual phospholipin shows a feeble inflexion at 275 mμ., and after chromatography the various fractions exhibit $E^{1\%}_{1\,cm.}$ (275 mμ.) 23·6–32·4. The petroleum-soluble phospholipin (0·61 g.) yielded 0·063 g. of unsaponifiable matter with $\lambda_{max.}$ 290 mμ., $E^{1\%}_{1\,cm.}$ 40. Most of the ultraviolet absorption of the phospholipin was thus due to saponifiable constituents, but a small amount of material which does not give a colour with the $SbCl_3$ reagent is present and remains to be accounted for.

Although the main point of this section is to show the need for eliminating phospholipins it is clear that they are likely to repay further study. It is possible that kitol may play a part in glyceride-phospholipin exchanges. It may well be that an investigation of the lipids of the absorptive portions of the whale intestinal tract would be worth while, in spite of the formidable difficulties of collecting and preserving the material.

Frozen whale liver as a source of oil

Large portions (50 kg.) of fresh livers of sperm whale (*Physeter catodon*, syn. *Physeter macrocephalus*) and blue whale (*Balaenoptera musculus*) were frozen right through in the Antarctic. The liver was brought to England in the ship's refrigerator and thereafter kept in a commercial cold store, and portions were chopped off as required.

Blue-whale liver. A portion (approx. 2 kg.) of liver was thawed, sliced and minced. The mince was extracted several times with cold acetone. The combined, concentrated, acetone extract was mixed with water and the fat extracted with light petroleum. The combined extracts were washed with water, dried over Na_2SO_4 and the solvent was removed. The product was 40 g. of yellow oil which became brown on standing; sp.gr. about 0·95; I_2 val. 163·4; saponification equiv. 330·8. The ultraviolet absorption curve showed $\lambda_{max.}$ 328 mμ., $E^{1\%}_{1\,cm.}$ 36·7 (gross), 29·8 after correction according to the procedure of Morton & Stubbs (1946). The curve (Fig. 2) was not very abnormal compared with that of a pure vitamin A ester. The kitol content was not high.

The liver debris was ground and re-extracted with ether-ethanol mixture (3 : 2 v/v). The filtered extract was diluted with water and re-extracted with ether. The solute (2 g.) contained very little vitamin A or kitol, but some decomposition products were present.

The oil from this sample of frozen blue-whale liver contained much less kitol than vitamin A, and there was relatively little interference with spectrophotometric determinations of vitamin A.

Sperm-whale liver. The oil obtained by extraction of minced liver with cold acetone was light brown in colour but darkened on standing; I_2 val. 149·8, saponification equiv. 378·5. The ultraviolet absorption curve showed a very flat maximum near 310 mμ., $E^{1\%}_{1\,cm.}$ (328 mμ.), 193·5; (286 mμ.) 122·9 (gross) (Fig. 3). The oil contained larger absolute amounts of vitamin A and kitol than that from blue-whale liver, and indeed a rough approximation indicated some 16% of vitamin A esters and perhaps 20% of kitol esters. The liver debris again gave a small yield of residual ether-soluble material.

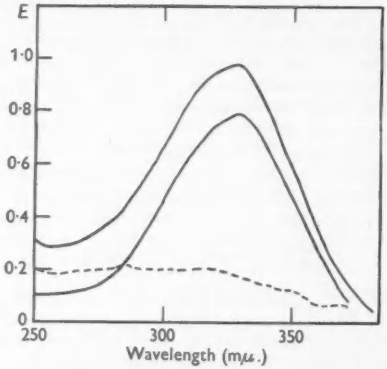

Fig. 2. Ultraviolet absorption spectrum of cold acetone extract of blue-whale liver $E^{1\%}_{1\,cm.}$ (328 mμ.) 36·7 (gross), 29·8 (corrected); solvent, *cyclo*hexane. Upper curve, observed; lower curve, corrected; broken line, subtraction curve.

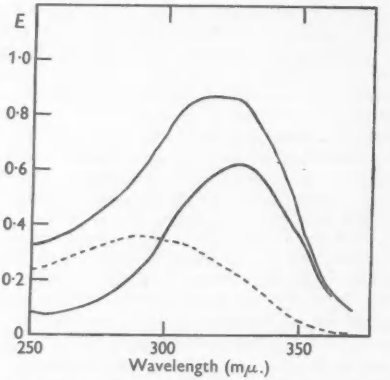

Fig. 3. Ultraviolet absorption spectrum of cold acetone extract of sperm-whale liver. $E^{1\%}_{1\,cm.}$ (328 mμ.) 193·5 (gross), 141·5 (corrected); solvent, *cyclo*hexane. Upper curve, observed; lower curve, corrected; broken line, subtraction curve.

The oil from this sample of sperm-whale liver was thus a good source both of vitamin A and kitol, but, as the latter was present in large amount, considerable interference occurred in the spectrophotometric determinations for vitamin A.

The experiments on well-preserved frozen livers suggest that in commercial whale-liver oils the presence of vitamin A decomposition products is a more serious analytical obstacle than the presence of kitol. This point is reinforced by the results of tests on salted minced whale livers. It is not necessary to go into all the experimental details, but it may be said that the oil samples obtained by various methods of extraction were fairly uniform, the $E^{1\%}_{1\,cm.}$ (328 mμ.) values (uncorrected) varying from 37 to 60. The percentage of unsaponifiable matter was about 25. Anticipating the discussion of the analytical problem (see p. 314), the fraction of the gross absorption at 328 mμ. due to vitamin A is about two-thirds and a conversion factor of 1200 is roughly correct. Attention is drawn at this stage to the fact that the oils obtained in the laboratory from preserved whale livers show the type of absorption spectrum to be expected from the summation of contributions due to kitol and vitamin A. From the point of view of analysis for vitamin A, the interference due to kitol is serious, but in commercial oils there is the additional difficulty of substantial irrelevant absorption due to artifacts.

Fractionation of whale-liver oils

Sand-column technique. Chevallier, Manuel & Faubert (1941) dispersed fish-liver oil on sand prior to extraction with solvents. The following experiment was carried out on the lines of their work: whale-liver oil concentrate (0·05 g.) was dissolved in 10 ml. redistilled ethyl ether, and the solution poured on to a column of fine, dry, silver sand (2 × 20 cm.). After allowing the solution to seep into the sand the ether was removed by a brisk upward current of N_2. The column was then washed with successive portions of solvent, with the results shown in Table 1. It was clear that 80% ethanol, if used in sufficient quantity, could extract anhydrovitamin A and vitamin A, leaving kitol behind.

Table 1. *Elution of whale-liver oil concentrate dispersed on sand*

Eluting solvent. Ethanol %	Volume (ml.)	$\lambda_{max.}$ (mμ.)	$E^{1\%}_{1\,cm.}$*	Vitamin A $E^{1\%}_{1\,cm.}$* 328 mμ. Gross	Vitamin A $E^{1\%}_{1\,cm.}$* 328 mμ. Corrected
		(*a*) 0·05 g. oil used			
70	270	328	—	42·4	33·6
80	540	328	—	50·8	26·6
90	90	292	17·6	—	—
100	90	290	25·3	—	—
		(*b*) 0·05 g. oil used			
60	90†	328	—	19·04	7·84
70	90†	328	—	9·65	6·44
80	90†	328	—	19·04	15·96
90	90	328	—	38·8	30·1
100	90	295	53·2	—	—

* Calculated on weight of original oil.
† Solvent passed through column five times.

A further experiment was tried on a larger scale and sand was not used: 0·5105 g. of 'oil' was boiled with 80% ethanol, the solution cooled and the supernatant liquor decanted. The 80% ethanol-insoluble fraction (0·3075 g.) showed $\lambda_{max.}$ 310–313 mμ. and contained about 4% vitamin A and 14% kitol. The soluble fraction (0·199 g.) showed $\lambda_{max.}$ 328 mμ. and contained some 3% of vitamin A contaminated by anhydrovitamin A and free kitol. The 'insoluble' fraction was repeatedly washed with hot 80% ethanol, cooled and the supernatant liquid decanted. The residue showed $\lambda_{max.}$ 295 mμ., $E^{1\%}_{1\,cm.}$ 125, corresponding with some 18% of kitol or about 36% kitol esters. This fraction (0·16 g.) was boiled with considerable excess of ethanolic KOH and 0·1044 g. unsaponified residue was obtained containing about 4% vitamin A and 23% 'free' kitol. This material was chromatographed on full-strength alumina (Spence, Grade 0) using light petroleum as the developing solvent. The various fractions were examined spectrophotometrically (Table 2). If $E^{1\%}_{1\,cm.}$ (286 mμ.) = 700 for pure kitol the material from the top zone contained 84% kitol (cf. Fig. 4).

Table 2. *Chromatography of* 0·08 *g. of unsaponifiable material from the fraction of whale-liver oil concentrate insoluble in* 80% *ethanol*

Zones	Wt. obtained (g.)	$\lambda_{max.}$ (mμ.)	$E^{1\%}_{1\,cm.}$ (286 mμ.)	$E^{1\%}_{1\,cm.}$ (286 mμ.) with respect to unsaponifiable material
Top	0·0105	286	588	77·20
Second	0·0096	286	317·1	39·63
Third	0·0564	325	21·3	14·90
Fourth	0·0019	—	—	0·75
—	0·0784	—	—	132·48

The foregoing experiments suffer from the disadvantage of being on a very small scale. In an attempt to overcome this, 50 g. of oil were heated with absolute ethanol (1 l.) until the solution became clear, and water (250 ml.) was then added and the solution cooled. The mixture was then put through a Sharples centrifuge. The soluble portion showed $\lambda_{max.}$ 326·5 mμ. and contained most of the vitamin A and anhydrovitamin A. The insoluble portion was repeatedly extracted with small amounts of ethanol. The residue was chromatographed in light-petroleum solution on full strength alumina. After several chromatographic separations it became clear that under these conditions fractions showing $\lambda_{max.}$ 286 mμ., $E^{1\%}_{1\,cm.}$ 166 were the best obtainable.

Chromatography of whole oil on a small scale gave two main kitol fractions, $\lambda_{max.}$ 286–290 mμ., $E^{1\%}_{1\,cm.}$ about 130 and 315, respectively, and the following decreasing order of adsorbability was indicated: free kitol, free vitamin A, kitol esters, vitamin A esters, anhydrovitamin A. Working on a 20–25 g. scale, fractions showing $E^{1\%}_{1\,cm.}$ about 160 at 295 mμ. were regularly obtained, but they were not free from vitamin A. One such fraction was chromatographed on weakened alumina (Spence, Grade 0, stirred with 10% (w/w) water). A fraction containing practically all the vitamin A was quickly obtained when the column was developed with light petroleum and was followed by a fraction showing $E^{1\%}_{1\,cm.}$ 367·5 at 286 mμ. and a further fraction exhibiting $E^{1\%}_{1\,cm.}$ 250 at 280 mμ.

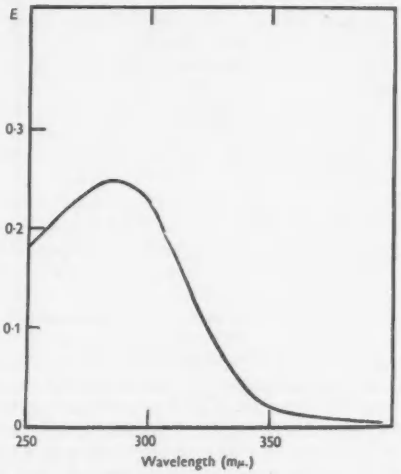

Fig. 4. Ultraviolet absorption spectrum of portion of whale-liver oil insoluble in 80% (v/v) aqueous ethanol. Unsaponifiable fraction chromatographed, strongly adsorbed zone at top of column; $E^{1\%}_{1\,cm.}$ (286 mμ.) 588 in *cyclo*hexane.

Attempts to crystallize kitol esters from crude preparations in acetone at low temperatures gave products of $\lambda_{max.}$ 280–286 mμ., $E^{1\%}_{1\,cm.}$ 160. Chromatography on '5% weakened' alumina gave two fractions $E^{1\%}_{1\,cm.}$ 168 and 349·2, respectively.

Chromatography of cold acetone extract of frozen sperm-whale liver

A portion of oil (4·18 g.), dissolved in light petroleum (25 ml.), was put through a column of full-strength alumina and developed first with light petroleum (1 l.) and then with light petroleum containing increasing amounts of acetone. The eluates (1–7) were examined spectroscopically with the following results: (1) Light brown oil, discarded. (2) $\lambda_{max.}$ 328 mμ., $E^{1\%}_{1\,cm.}$ 254; some anhydrovitamin A was present. (3) $\lambda_{max.}$ 328 mμ., $E^{1\%}_{1\,cm.}$ 282; absorption curve practically normal for vitamin A. (4) $\lambda_{max.}$ 290–295 mμ., $E^{1\%}_{1\,cm.}$ (286 mμ.) 253, gave no vitamin A band at 620 mμ. in the colour test. (5) Contained some vitamin A alcohol, $E^{1\%}_{1\,cm.}$ (328 mμ.) 82. (6) $\lambda_{max.}$ 328 mμ., $E^{1\%}_{1\,cm.}$ 152, practically normal vitamin A curve (free alcohol). (7) Brown portion of column cut out, eluted with ether; fraction was mainly kitol (free), $E^{1\%}_{1\,cm.}$ (286 mμ.) 152.

Fraction 4 was rechromatographed on full-strength alumina and developed with light petroleum. Two main zones appeared and three portions were cut out from the middle of the lower zone. All three were eluted with ether. The ether

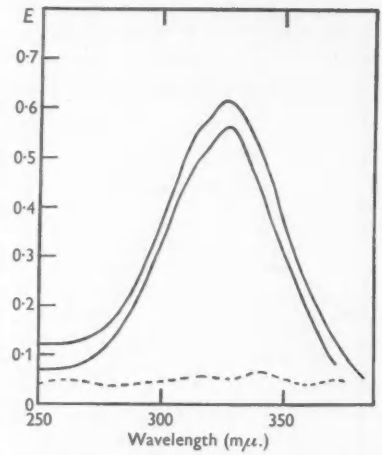

Fig. 5. Chromatography of phospholipin-free, neutral, sperm-whale-liver oil. Ultraviolet absorption spectrum of vitamin A ester fraction $E^{1\%}_{1\,cm.}$ (328 mμ.) 305 (gross), 280 (corrected) [or 112·8 (corrected) with respect to oil]; solvent, *cyclo*hexane. Upper curve, observed; lower curve, corrected; broken line, subtraction curve.

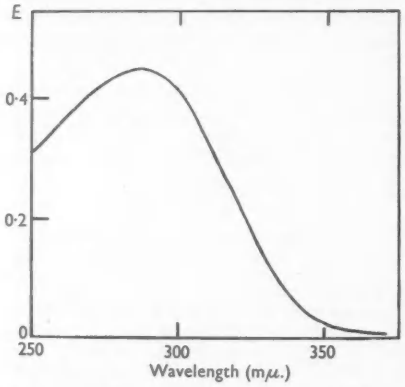

Fig. 6. Ultraviolet absorption spectrum of kitol ester fraction, 0·0013% in *cyclo*hexane, 1 cm. $E^{1\%}_{1\,cm.}$ (286 mμ.) 346.

was removed and the weighed residues were tested in *cyclo*hexane. The absorption curves were very flat over the range 286–290 mμ. and $E^{1\%}_{1\,cm.}$ values of 166, 343 and 353 were obtained.

A large number of experiments on sperm-whale-liver oil indicated that in seeking to prepare pure kitol esters it is desirable to use material free from phospholipins or free fatty acids. The following method is perhaps the simplest: To sperm-whale-liver oil acetone is added and, after allowing phospholipins to settle out, the solution is filtered through a bed of kieselguhr mixed with solid $NaHCO_3$ (used as a fine powder). The filtrate is diluted with water and extracted with light petroleum, washed with water, dried over Na_2SO_4, filtered and the solvent evaporated. The oil (approx. 10 g.) is dissolved in light petroleum and chromatographed on alumina weakened by addition of 10% (w/w) of water. Light petroleum is used at first to develop the chromatogram (Fig. 5). The eluate is periodically tested with the $SbCl_3$ reagent. When the vitamin A colour test becomes weak, development is continued with light petroleum containing 5% ether (v/v). When kitol esters begin to be eluted a purple colour will be obtained with the $SbCl_3$ reagent. In two such experiments fractions showing $E^{1\%}_{1\,cm.}$ (295 mμ.) 346 (Fig. 6) were obtained weighing 0·85 and 0·52 g. respectively.

The best preparations of kitol (esterified) were all in agreement with the figure of $E^{1\%}_{1\,cm.}$ about 350 as the highest attainable.

Saponification of kitol esters

The results of attempts to prepare pure kitol by saponifying the esters and extracting the unsaponifiable fraction were erratic and rather disappointing. Thus one ester preparation

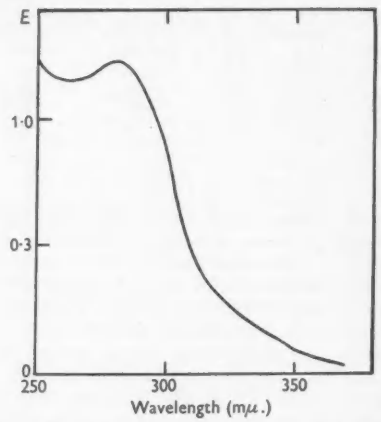

Fig. 7. Ultraviolet absorption spectrum of kitol ester fraction, unsaponifiable matter after two treatments with alkali; $E^{1\%}_{1\,cm.}$ (286 mμ.) 230.

showing $\lambda_{max.}$ 286 mμ., $E^{1\%}_{1\,cm.}$ 350 gave 54·8% of unsaponifiable matter with $\lambda_{max.}$ 280 mμ., $E^{1\%}_{1\,cm.}$ 156, but the kitol had undoubtedly decomposed. The recovered acid amounted to 43% of the material used. Another good ester preparation, left to stand in the cold with ethanolic KOH for 48 hr., resisted saponification. On treatment with ethanolic KOH at the boiling point in a current of N_2 the $E^{1\%}_{1\,cm.}$ value of the unsaponifiable fraction rose to 410, but fell to 230 ($\lambda_{max.}$ 280 mμ.) after retreatment with alkali for a longer time on the steam bath (Fig. 7). The recovered acids (33%) had a mean mol. wt. of 284.

With another preparation the total recovered fatty acids accounted for 48·3 % of the kitol ester fraction. The following illustrates some of the later experiments: 0·771 g. kitol ester fraction, $E^{1\%}_{1\,cm.}$ (286 mμ.) 346 was dissolved in ethanol (10 ml.) and 40 % aqueous KOH (4 ml.) was added. A little quinol was used as antioxidant. After boiling on the water bath until the solution was quite clear, the unsaponified material was extracted in the usual way and the fatty acids recovered from the soaps. The $E^{1\%}_{1\,cm.}$ value at 285 mμ. had risen to 432 and 0·296 g. of acids was recovered. Resaponification with sodium ethoxide (boiling for 15 min.) raised the $E^{1\%}_{1\,cm.}$ value of the non-saponifiable material to 620 and gave a further 0·125 g. of acids.

If kitol has a mol. wt. of 572 and an $E^{1\%}_{1\,cm.}$ (286 mμ.) of 700, then an $E^{1\%}_{1\,cm.}$ value of 350 for mixed kitol esters implies that these are diesters of acids with an average mol. wt. of about 304.

Pyrogenic decomposition of kitol esters

When heated carefully, kitol esters darken in colour, and the product shows weakly a maximum at 620 mμ. in the $SbCl_3$ colour test. Small quantities (0·02 g.) heated electrically in capillary tubes showed no decomposition at temperatures below 200°. From 230 to 250° (4–5 min.) faint positive tests were obtained ($SbCl_3$ max. 612–620 mμ.), but the yield of vitamin A from the pyrogenic decomposition of the esters was poor. The special conditions of molecular distillation are clearly needed for good yields.

Administration of kitol esters to the avitaminotic rat

When given as 0·12 g. in 1 ml. of coconut oil spread over 2 days and administered by stomach tube, kitol esters were neither converted into vitamin A nor appreciably stored in the liver 40 hr. after the second dose.

A chromatographic method for the determination of vitamin A in whale-liver oils

The experiments leading to the isolation of a kitol ester fraction suggested a new approach to the analytical problem. The matter has a somewhat complicated history. At an early period in the history of the use of whale-liver oils as a source of vitamin A a commercial concentrate was studied (Edisbury *et al.* 1935, 1937) which exhibited anomalous ultraviolet absorption ($\lambda_{max.}$ about 295–305 mμ.) and an anomalous colour test as compared with fish-liver oils. It was found empirically that by measuring $E^{1\%}_{1\,cm.}$ (328 mμ.) and multiplying by 1200, estimates of potency in reasonably good agreement with the results of bioassays were obtained.

With the resumption of whaling after the end of the War it became necessary to determine vitamin A in crude oils extracted on board the factory ships. It speedily emerged in these laboratories, and in those of Lever Brothers and Unilever Ltd. at Port Sunlight, that neither the colour test nor the ultraviolet absorption was anomalous in a constant fashion, and the basis for the empirical use of a conversion factor of 1200 instead of 1600 (widely used for fish-liver oils) disappeared. The correction procedure of Morton & Stubbs (1946) was often stretched beyond the limits of its utility by the magnitude of the irrelevant absorption. It had also become clear that the factor of 1600 was itself a reflexion of the fact that, on the average, fish-liver oils exhibited some 11 % or so of 'irrelevant' absorption and that a factor of 1800 was appropriate to preparations in which vitamin A was the only significantly absorbing entity (Morton & Stubbs, 1947). The problem of analysing whale-liver oils thus resolved itself into the task of obtaining all the vitamin in a fraction (or fractions) free from other substances absorbing near 328 mμ. The following experiments indicate how the problem has been solved.

Sperm-whale-liver oil (1·713 g.) was dissolved in 15 ml. light petroleum in a small conical flask and powdered $NaHCO_3$ (1 g.) added, and the mixture thoroughly shaken. Weakened alumina was prepared by placing aluminium oxide (50 g., Spence, Grade 0) in a mortar, covering with light petroleum and adding water (5 ml.). The mixture was stirred thoroughly with a pestle and used to fill a tube 44 × 1 cm. A layer (1·5 cm. high) of powdered $NaHCO_3$ was put on top of the column. The oil in light petroleum was then poured on to the column without disturbing the sediment. The residual contents of the conical flask were washed with light petroleum several times and the solution poured on to the column. Light petroleum was used to develop the chromatogram. A light yellow band travelled down the column and the eluate was collected in 20 ml. portions, a drop from each of which was tested with the $SbCl_3$ reagent. The portions were sorted out into three groups: (*A*) those giving a normal vitamin A colour test, (*B*) those giving a purple colour and, finally, (*C*) those obtained only when the developing solvent contained ether (10 or 20 % by vol. in

Table 3. *Chromatographic separation of constituents of sperm-whale-liver oil*

(Wt. of oil, 1·713 g.)

Fractions	Wt. recovered (g.)	$\lambda_{max.}$ (mμ.)	$E^{1\%}_{1\,cm.}$	$E^{1\%}_{1\,cm.}$ with respect to oil	$SbCl_3$ colour test ($\lambda_{max.}$)
A	0·517	328	(328 mμ.) 316 gross 280 corr.	(328 mμ.) 96 gross 84 corr.	620 mμ. (blue)
B	0·364	300	(300 mμ.) 218·5 gross (328 mμ.) 34 corr. (286 mμ.) 233 corr.	(328 mμ.) 7·2 corr. (286 mμ.) 49·4	(purple), 495 mμ., 505 mμ., 580 mμ.
C	0·064	280	(280 mμ.) 38 gross	—	(Red)

light petroleum). The solvent was removed from the three fractions and the residues weighed and subjected to spectroscopic analysis (Table 3).

It is evident from Table 3 that fraction *A* contained most of the vitamin A and was easily analysed, *B* contained most of the kitol, and *C* consisted mainly of decomposition products. A further experiment was carried out to try to improve the separation.

Sperm-liver oil (0·998 g.) and $NaHCO_3$ (0·5 g.) were shaken with light petroleum and the solution chromatographed on weakened alumina as before. The eluate was collected in 5 ml. portions and those which gave a normal vitamin A blue with the $SbCl_3$ reagent were bulked, the solvent removed and the residue weighed ($0{\cdot}282_4$ g.). This was made up to volume with *cyclo*hexane and a measured portion was diluted for spectrophotometry. The absorption maximum occurred at 328 mμ. with $E^{1\%}_{1\,cm.}$ 100·8 (gross) calculated on the weight

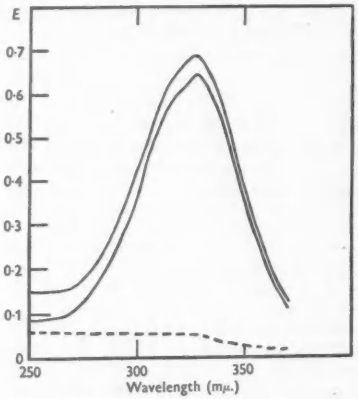

Fig. 8. Chromatographic analysis of sperm-whale-liver oil. Ultraviolet absorption spectrum of vitamin A ester fraction; $E^{1\%}_{1\,cm.}$ (328 mμ.) 100·8 (gross), 94·4 (corrected) with respect to oil; solvent, *cyclo*hexane. Upper curve, observed; lower curve, corrected; broken line, subtraction curve.

of oil used (Fig. 8). This value was reduced to 94·4 on applying the three-point correction procedure for irrelevant absorption. The previous experiment gave 84 + 7·2 = 91·2, but the vitamin was not all in the first fraction. It will be seen that by collecting the eluate in 5 ml. portions, one drop of each of which is tested with $SbCl_3$, a very clear-cut separation can be achieved.

The procedure needs, however, to be subjected to a more severe test. Accordingly, a sample of low-potency oil of inferior quality was tested.

Crude whale-liver oil (0·2665 g.) in light petroleum (20 ml.) was chromatographed on a column of weakened alumina 15 × 1 cm. and developed with light petroleum. The eluate was collected in 5 ml. portions. The first few portions contained anhydrovitamin A (pale greenish yellow; blue colour with $SbCl_3$). The next four portions gave no colour with the $SbCl_3$ reagent and were discarded. Subsequent portions contained vitamin A (esters) giving a normal colour test. Elution was continued until the solution was no longer chromogenic towards $SbCl_3$. The column was then eluted with ether. The vitamin A fraction was made up to 50 ml. with light petroleum and a measured portion diluted with *cyclo*hexane so that the concentration was 0·0533 % with respect to the original oil. It gave $\lambda_{max.}$ 328 mμ., $E^{1\%}_{1\,cm.}$ 8·74 gross, 8·4 (corr.). The ether eluate was made up to 100 ml. with light petroleum, and a measured portion diluted with *cyclo*hexane to 0·02665 % with respect to the original oil. It gave $E^{1\%}_{1\,cm.}$ (286 mμ.) 12·5 and contained kitol esters together with decomposition products, but no vitamin A was detectable. (If at any time the percolation of solvent through the column becomes too slow, it is advisable to stir the adsorbent at the top with a glass rod.)

To test the method further a more complete analysis was made on another sample of crude oil. Crude whale-liver oil (0·2 g.) in light petroleum (20 ml.) was poured on a column (15 × 1 cm.) of weakened alumina, and the chromatogram developed with light petroleum followed by the same solvent containing increasing proportions of ether. The percolate was collected in 5 ml. portions, 1 drop of each being tested with the $SbCl_3$ reagent.

The following fractions were obtained: (1) Anhydrovitamin A, discarded. (2) Vitamin A esters; 180 ml. of eluate $\lambda_{max.}$ 328 mμ., $E^{1\%}_{1\,cm.}$ 14·6 (gross), 14·14 (corrected); *E* values calculated on weight of oil taken. (3) Kitol ester fraction; elution complete after using light petroleum containing 4, 8 and 12 % ether. $\lambda_{max.}$ 290 mμ., $E^{1\%}_{1\,cm.}$ 8·125. (4) Fraction containing free vitamin A and some free kitol; $\lambda_{max.}$ 326 mμ., $E^{1\%}_{1\,cm.}$ 4·22 (gross), 3·02 (corr.); elution completed with 12, 16 and 20 % ether in petrol. (5) Fraction eluted with ether; no vitamin A present as judged by colour test.

For this oil $E^{1\%}_{1\,cm.}$ (328 mμ.; gross) was 28·7 and the net contribution due to vitamin A was 14·14 + 3·02 = 17·16. The simple correction procedure of Morton & Stubbs (1946) gave 15·8—a figure which is significantly low.

Similar experiments have led to the following standard procedure.

Analysis of commercial whale-liver oil

Adsorbent. Alumina (Spence, Grade 0) for adsorption (100 g.) is made into a slurry with light petroleum in a glass mortar, and water (10 ml.) is added and the whole mixed with a pestle for 5 min. The slurry is then poured into an adsorption tube and allowed to settle. The weakened adsorbent should be used the same day.

Column. The column of weakened alumina should be 12 × 1 cm. if the weight of oil to be chromatographed is 0·2 g. The sample is dissolved in light petroleum (25 ml.) and poured on to the column. A large bottle (15–20 l.) is fitted with a rubber bung with two holes, through which tubes, each with a tap, are inserted; one is connected by a rubber tube to a bicycle pump and the other to a short piece of glass tubing in a rubber bung fitting the top of the

adsorption apparatus. Passage of solvent through the column is thus hastened by pressure from above, and percolation may be quite rapid (approx. 2 ml./min.). There should be room for a least 25 ml. of solvent above the absorbent.

Development. The chromatogram is developed first with light petroleum and the percolate collected in small test tubes graduated at 5 ml. One drop from each portion is tested with 0·5 ml. of $SbCl_3$ reagent. The first few portions will give no colour and may be discarded. Then anhydrovitamin A will appear in the eluate; it gives a blue colour with the $SbCl_3$ reagent and, after a volume of 35–40 ml. has passed through the column, no colour will appear. (The volume will, of course, vary somewhat from sample to sample.) Continued development with light petroleum results in the appearance of vitamin A esters in the eluate. The intensity of the blue colour obtained with one drop of successive portions of percolate will at first increase and finally decrease until no colour is given. By this time, 100–200 ml. of liquid will have passed through the column. Development is continued at first with light petroleum containing 4 % ether (by vol.) and then with 8 and with 12 % ether-petroleum mixtures, usually 50 ml. portions at each strength. The percolate at this stage gives a purple colour with the $SbCl_3$ reagent and exhibits an ultraviolet absorption maximum near 285–290 mμ. A small volume of eluate then appears which is practically non-chromogenic towards $SbCl_3$. Development with ether-petroleum mixtures (12, 16, 20 % by vol.) is continued, and fractions are obtained giving blue and purple colours with $SbCl_3$, indicating free vitamin A and free kitol respectively. The 5 ml. portions of eluate are grouped and the separate fractions each made up to a definite volume and examined by photoelectric spectrophotometry.

Example. $E^{1\%}_{1\,cm.}$ (328 mμ.) 60·1 on the whole oil. 0·125 g. examined as above:

(i) Anhydrovitamin A fraction $E^{1\%}_{1\,cm.}$ (370 mμ.) 1·6.
(ii) Vitamin A ester fraction, made up to 200 ml., diluted 5 ml./20 ml. *cyclo*hexane. $E^{1\%}_{1\,cm.}$ (328 mμ.) 37·9, no correction needed.
(iii) Kitol ester fraction, $\lambda_{max.}$ 285–290 mμ. $E^{1\%}_{1\,cm.}$ 24·35.
(iv) Free vitamin A fraction, $E^{1\%}_{1\,cm.}$ (328 mμ.) approx. 2·2 corr.

Total $E^{1\%}_{1\,cm.}$ (328 mμ.) 40 (i.e. 37·9 + 2·1).

Estimated potency $40 \times 1800 = 72{,}000$ i.u./g.

Separation of vitamin A from whale-liver non-saponifiable matter by means of 50 % ethanol

By washing the unsaponifiable fraction of whale-liver oil with 50 % (v/v) aqueous ethanol, free vitamin A is preferentially dissolved. Under certain circumstances this forms the basis of a useful analytical procedure.

Example. A commercial whale-liver oil concentrate (0·5 g.; $E^{1\%}_{1\,cm.}$ (328 mμ.; 210 gross) was saponified, and the unsaponifiable fraction showed $E^{1\%}_{1\,cm.}$ 212·6 at 326 mμ. (calculated on the wt. of raw material). The absorption curve was, however, anomalous and there was evidence of considerable irrelevant absorption. The unsaponifiable matter was shaken well with 50 % ethanol (100 ml.), and the supernatant liquid filtered carefully through a small bed of kieselguhr (filter aid). Six further washings (2 × 100 ml. and 4 × 50 ml.) were filtered in the same way. The combined filtrate was made up to 500 ml. (*A*). The flask and the filter aid were then washed repeatedly with small quantities of redistilled ether. The solvent was removed and the extract dried by treatment with ethanol in a current of N_2 on the water bath; the residue was dissolved in ethanol and the volume made up to 100 ml. (*B*).

A measured volume of solution *A* was diluted with ethanol and showed $\lambda_{max.}$ 326 mμ., $E^{1\%}_{1\,cm.}$ 135·8 (corrected by the Morton & Stubbs (1946) method). Solution *B*, suitably diluted, gave $\lambda_{max.}$ 287 mμ., $E^{1\%}_{1\,cm.}$ 85 (*E* values calculated with respect to the original oil; see Fig. 9).

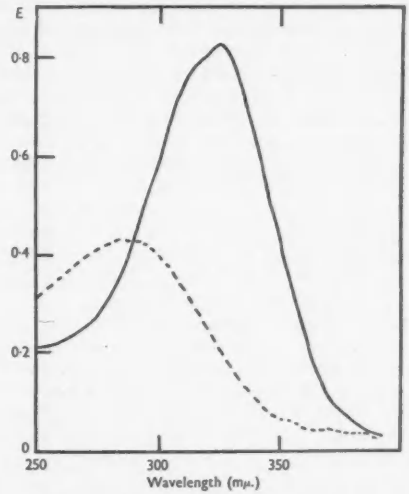

Fig. 9. Ultraviolet absorption spectrum. —— Portion of whale-liver oil unsaponifiable material soluble in 50 % (v/v) aqueous ethanol; $E^{1\%}_{1\,cm.}$ (326 mμ.) 164 (gross), 135·8 (corrected), calculated for original oil; solvent, ethanol. ---- Portion of unsaponifiable material insoluble in 50 % ethanol; $E^{1\%}_{1\,cm.}$ (287 mμ.) 85; solvent ethanol.

Further trials showed that most, but not all, of the irrelevant absorption can be eliminated from whale-liver oil unsaponifiable fractions by extraction with 50 % aqueous ethanol. The kitol fraction is relatively insoluble in this medium. Judged, however, by the strictest standards, the method is imperfect, because when an oil is contaminated with artifacts, as well as with kitol, a little extraneous

material absorbing at 326 mμ. is liable to accompany the vitamin A alcohol. The test, however, often affords valuable confirmation (see Table 4).

Table 4. *Correction of absorption of a whale-liver oil concentrate*

(Concentrate had $E^{1\%}_{1\,cm.}$ (328 mμ.) 210 (gross))

	E (corr.)	Vitamin A
Concentrate	141·4	*Assuming it to be all esterified
	128·8	*Assuming it to be free
Unsaponifiable extract	130	*Assuming it to be free
50% ethanol extract	135·8	*Assuming it to be free

* The absorption curves for vitamin A alcohol and esters are not quite identical.

DISCUSSION

Numerous preparations from samples of whale-liver oil have been obtained showing an absorption maximum at 285–295 mμ. with $E^{1\%}_{1\,cm.}$ 346–360, and further chromatography has failed to raise the intensity of absorption. The preparations were practically free from glycerides or vitamin A. They underwent saponification with difficulty, but finally yielded approximately half their weight as recovered fatty acids after extracting the acidified soaps with ether. The unsaponifiable material tended to decompose with consequent loss of absorption intensity, but in one case an $E^{1\%}_{1\,cm.}$ (286 mμ.) value of 620 was obtained when quinol was used as antioxidant during the saponification. Kitol, which has been reported as possessing an $E^{1\%}_{1\,cm.}$ value of 700 when obtained as freshly recrystallized material, is not very stable, even in the solid state in sealed ampoules; it does not withstand chromatography without decomposition and it is unstable towards alkali. If it is re-esterified, the esters are sometimes recrystallizable, but *cis-trans* isomerism may bring in complications.

The 'purified' kitol preparations of $E^{1\%}_{1\,cm.}$ 350 are clearly mixed diesters containing an assortment of higher fatty acids of molecular weights exceeding 280. The preparation of such kitol esters appears to rule out the possibility that kitol is an artifact, and to show that kitol esters are present in whale liver. If the samples of blue- and sperm-whale livers were representative, kitol esters are quantitatively much more significant in sperm whales than in blue whales. Too little, however, is known about variations between individual whales of either species to permit this observation to be generalized.

The fact that kitol is a di(vitamin A) is fully established, and from its absorption spectrum it must have a chain (or two chains) of four conjugated double bonds instead of the five conjugated double bonds of vitamin A. The molecular extinction coefficient of kitol is about 41,000 and that of vitamin A about 50,000.

Gridgeman, Savage & Gibson (1948), working at the Port Sunlight laboratories of Lever Bros. and Unilever Ltd., have for some time been engaged upon the problem of determining vitamin A in whale-liver oils. They have worked out an excellent method for the separation of vitamin A alcohol from unsaponifiable extracts by chromatography, and we are indebted to Messrs Lever Bros. for information and advice over a long period.

Comparing the method we have described with the procedure based on preliminary saponification it seems likely: (*a*) that for oils in which much of the vitamin A is free there are advantages to be gained from completing the saponification in aiming at an estimate of the vitamin A content at the time of the test; (*b*) that for oils in which most of the vitamin A is esterified, direct chromatography is preferable in minimizing losses and in speed of operation; (*c*) that a determination of free and esterified vitamin A gives some indication of the probable stability of the oil, as it is the free vitamin A which forms anhydrovitamin A and probably some artifacts.

SUMMARY

1. The acetone-soluble lipid from a sample of frozen blue-whale liver contained vitamin A ($E^{1\%}_{1\,cm.}$ (328 mμ.) 36·7) with relatively little kitol. A similar extract from frozen sperm-whale liver was richer in vitamin A and kitol (16 and approx. 20%, respectively, as esters).

2. The fractionation of whale-liver oils has been attempted in various ways. Free vitamin A is extracted by means of 50% aqueous ethanol, and vitamin A esters are separable from kitol esters by means of 80% aqueous ethanol.

3. Chromatography of whale-liver oil on alumina (weakened by addition of 10% (w/w) water) effects a clean separation of vitamin A (esterified) from kitol (esterified). Kitol ester fractions showing $E^{1\%}_{1\,cm.}$ approx. 350 and approx. 170 at 286 mμ. were regularly obtained.

4. Kitol esters are difficult to saponify, and the free kitol tends to be decomposed unless precautions are taken. They contain, however, about half their weight of kitol. $E^{1\%}_{1\,cm.}$ (286 mμ.) values of about 620 have been obtained for free kitol.

5. Kitol esters fed to rats are neither converted to vitamin A nor stored in the liver.

6. A chromatographic method for the determination of vitamin A in unsaponified whale-liver oils has been described and tested.

7. A fairly good separation of kitol from vitamin A in whale-liver unsaponifiable fractions can be effected by extracting the vitamin A with 50% aqueous ethanol, but the volume used limits the method to analytical work.

8. Preliminary work on whale-liver phospholipins suggests that kitol is not present in the true acetone-insoluble lipid, but that it may occur in more than one type of combination.

9. Vitamin A and possibly kitol is present in fin-whale milk.

A small sample of kitol was kindly placed at our disposal by Dr Baxter of Distillation Products Inc. We are indebted to the Ministry of Food and the Medical Research Council for grants in aid of the work. In obtaining material we have had most friendly help from the whaling industry and many scientists engaged in it both in this country and abroad.

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Studies in Vitamin A

13. THE ALLEGED FORMATION OF VITAMIN A FROM β-CAROTENE TREATED WITH IODINATED CASEIN

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Kaplansky & Balaba (1946) claimed that β-carotene in colloidal solution when incubated with either thyroglobulin or iodinated casein was converted into vitamin A in good yield; a typical experiment resulted apparently in the production of 60 μg. of vitamin A from 150 μg. of β-carotene. If it be true that thyro-active compounds can effect such a conversion, the finding is of major importance to the biochemistry of carotene and vitamin A. An investigation of Kaplansky & Balaba's claim was therefore undertaken. In the first part of the work the experimental procedure of Kaplansky & Balaba was followed as closely as possible, but when efforts to produce vitamin A failed, the conditions were then varied. A short report of the results of this work has already appeared (Cama & Goodwin, 1948).

EXPERIMENTAL

Preparation of iodinated casein. $NaHCO_3$ (3·6 g.) was added to skimmed milk (500 ml.) freed from fat by repeated centrifugation, and the liquid heated to 40°. Small portions of I_2 were added with constant stirring until a slight excess remained at the bottom of the beaker (about 8·0 g. of I_2 were required). The temperature was then raised to 65° for 24 hr., the liquid being constantly stirred. After cooling, the intensely yellow solution was filtered and the filtrate brought to pH 4·5 with acetic acid. The precipitated iodinated casein was washed, dissolved in very dilute alkali and reprecipitated with acetic acid. This process was repeated three times. The I_2 content of the product, determined according to the micro method of Groák (1926) was 8·45–8·67%. Kaplansky & Balaba (1946) report 0·85%, but this appears to be a mistake.

Preparation of colloidal solutions of β-carotene. Crystalline β-carotene (approx. 3 mg.) was dissolved in a small volume of freshly redistilled diethyl ether (20 ml.) and 96% (v/v) ethanol added (5 ml.). The ether was removed by distillation on a water bath at 50–60°; the distillation was continued until a test portion of the distillate no longer separated into two layers after the addition of a small volume of water. The ethanol solution of β-carotene was then diluted with distilled water and rapidly filtered; the colloidal solution was placed in a boiling water bath to remove the ethanol and any residual ether. The remaining concentrated colloidal solution was filtered again, diluted with water to approximately the desired concentration and used without delay.

Incubation of solutions. The details of Kaplansky & Balaba's (1946) method are as follows: To a known volume of colloidal solution containing 150 μg. β-carotene is added iodinated casein (30 μg.) dissolved in weak alkali; the reaction mixture, after being adjusted to pH 7·3 with acetic acid, is incubated for 2 hr. at 37°; controls consisting of β-carotene solutions only or β-carotene solution + boiled iodinated casein adjusted to pH 7·3 are treated in the same way.

In the first stages of the present investigation the above procedure was followed as closely as possible. (Kaplansky & Balaba did not specify the concentration of the weak alkali or the acetic acid; we used 0·01 N-$NaHCO_3$ and 0·01 N-HOAc.) The procedure was later modified by altering (*a*) the pH (by the use of phosphate buffers), (*b*) the incubation times, and (*c*) the amounts of substrate and 'enzyme', and (*d*) by using desiccated thyroid or minced fresh thyroids instead of iodinated casein.

Determination of carotene and vitamin A. Kaplansky & Balaba used the method of Rachevski for the determination of carotene; no reference or details were given, but a search of the literature indicates that the method used can only be that of Rachevski & Troitski (1938). It is not based on absorption spectrophotometry as are most methods of carotenoid analysis, but on iodometry. A known amount of iodine is added to a colloidal solution of carotene and the amount not absorbed is measured in the usual way by titrating with thiosulphate. Such a method, as will be noted later, has serious drawbacks when used in experiments of the type under discussion. We preferred the method in use in this laboratory, as it is unambiguous and well authenticated.

A measured volume of the colloidal solution is extracted three times with an equal volume of freshly redistilled diethyl ether; the ether extracts are combined and the solvent removed on the water bath; the residue is dissolved in a known volume of *cyclo*hexane and the absorption spectrum recorded using a Beckman photoelectric spectrophotometer. *cyclo*Hexane is chosen as the solvent so that vitamin A determinations, making use of both ultraviolet absorption and the $SbCl_3$ colour test, could be carried out on the same solution; this eliminates further manipulations involving heat, and minimal heat treatment is always to be aimed at when dealing with carotenoids.

Kaplansky & Balaba stated that they determined vitamin A by the Carr-Price method ($SbCl_3$ reagent), and, in one experiment, by the spectrophotometric determination of the absorption maximum at 328 mμ., but no details of the method were given. In the present investigation two procedures for determining vitamin A were used: (*a*) the Morton & Stubbs (1946) method of direct ultraviolet spectrophotometry, and (*b*) the $SbCl_3$ colour test. The Morton & Stubbs method is a well-authenticated method in which allowance can be made for absorption not due to vitamin A. The $SbCl_3$ colour test was carried out using a Hilger-Nutting visual spectrophotometer; this instrument allows the differentiation of the $SbCl_3$-vitamin A absorption maximum at 617 mμ. from $SbCl_3$-carotene absorption maximum at 590 mμ. even when carotene occurs in considerable excess over vitamin A.

RESULTS

Thirty-six experiments were carried out under varying conditions, and in no case was the formation of vitamin A unequivocally demonstrated after incubation of β-carotene with either iodinated casein, desiccated thyroid, or fresh thyroid brei; in one case only the Morton & Stubbs (1946) correction procedure pointed to the presence of a trace of vitamin A, but this was not confirmed by the antimony trichloride colour test.

In the first series of experiments Kaplansky & Balaba's (1946) conditions were reproduced as closely as the published directions permitted; later the conditions were varied and Table 1 indicates the limits of variation.

In Fig. 1 are shown the full absorption curves obtained in three typical experiments in which β-carotene was incubated: (*a*) alone, (*b*) with iodinated casein, and (*c*) with desiccated thyroid. It will be noted that although the curves do not coincide, owing to the difficulty of obtaining colloidal carotene solutions of the same concentration (see p. 319), they are almost identical in shape; thus no differential changes in the β-carotene have occurred by incubation under different conditions. The appearance of a small band at 336 mμ. is due to *trans-cis* isomerization of the carotenoid produced under the action of heat; the 336 mμ. band is Zechmeister's (1944) *cis* peak.

Table 1. *Variations in the experimental conditions used in attempting to convert β-carotene into vitamin A by incubating with iodinated casein or desiccated thyroid*

Variable	Limits of variation
β-Carotene	2·5–30 μg.
Incubation time	2–2·5 hr.
pH	4–8
Iodinated casein	50–60 μg.
Desiccated thyroid	30–60 μg.
Temperature	Not altered (37°)

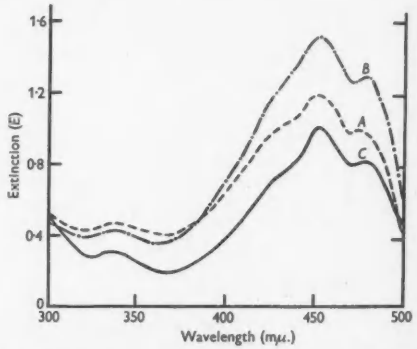

Fig. 1. The absorption spectrum of β-carotene: *A*, after incubation alone for 2·5 hr. at 37°; *B*, after incubation with iodinated casein for 2·5 hr. at 37°; *C*, after incubation with desiccated thyroid for 2·5 hr. at 37°.

DISCUSSION

It will be seen that we have not, in this laboratory, been able to detect the formation of vitamin A when colloidal solutions of β-carotene are incubated with iodinated casein or desiccated thyroid. The apparatus used is known to be capable of detecting small amounts of vitamin A in the presence of carotene so there is no doubt that vitamin A was not present; it was not a matter of failure to detect it.

Kaplansky & Balaba (1946), on the other hand, report three experiments in which the yield of vitamin A was 45%. We have thus to attempt a reconciliation between contradictory experiences. It may be that favourable conditions are elusive, and

that although we tried to follow Kaplansky & Balaba's methods exactly we failed to achieve such conditions; it seems, however, that a method which produces a 45 % yield of vitamin A in the hands of one set of workers should in another laboratory at least yield detectable amounts. It is interesting to note that Wiese-Lowry (1949) has informed us that she too was unable to repeat Kaplansky & Balaba's work. It is possible to criticize Kaplansky & Balaba's work from a technical viewpoint, and it is then clear that their claims are not securely based. Their technique may be considered under three heads:

(i) *Estimation of β-carotene.* The iodometric method used by Kaplansky & Balaba is at best unspecific, and it is difficult to understand why it was used if an optical instrument were available (as apparently there was, because in one experiment *E* 328 mμ. was measured). The iodometric method presents a problem which Kaplansky & Balaba have not discussed. For example, the results of a typical experiment recorded by them indicates that 150 μg. of β-carotene after incubation with iodinated casein yielded 86 μg. of β-carotene and 55 μg. of vitamin A. Now, vitamin A is an unsaturated compound and will itself absorb iodine; thus, unless this is allowed for, the iodometrically determined β-carotene content of a mixture of β-carotene and vitamin A will be incorrect. Assuming this correction has not been made, and there is no internal evidence that it has, the results just quoted are open to two serious objections: (*a*) If the β-carotene not converted into vitamin A is reasonably stable to incubation, as they claim it is in the case of boiled iodinated casein, and as we find it is in all cases, then the iodometric method should give a value for 'β-carotene' still in the neighbourhood of 150 μg. (*b*) If the value 86 μg. was the determined uncorrected value then this must represent 'β-carotene and vitamin A', and as 55 μg. of vitamin A are reported only about 30 μg. of β-carotene have been accounted for out of an expected remainder of about 100 μg.

(ii) *Colloidal carotene solutions.* Although following precisely the directions given by Kaplansky & Balaba, we found the production of the colloidal solutions to be much less reproducible than they did. For example, according to their table they used for each experiment exactly 150 μg. of carotene obtained by diluting a concentrated colloidal solution a known amount. We could not make this method work, for, no matter how carefully the water was added to the concentrated carotene solution, a little was always thrown out of solution and had to be filtered off; thus it was impossible to obtain a solution of colloidal carotene of known strength merely by dilution; the content of each colloidal solution had to be measured each time.

(iii) *Measurement of vitamin A.* Kaplansky & Balaba give no details of how they measured the $SbCl_3$ colour test. How they differentiated between the carotene-$SbCl_3$ colour test and the vitamin A-$SbCl_3$ colour test, especially in a mixture of two, is not stated, so that it is impossible to assess the evidence.

Regarding the determination of vitamin A in the presence of excess of β-carotene by ultraviolet spectrophotometry, β-carotene itself absorbs in the 300–340 mμ. region and this has to be allowed for before the contribution of vitamin A to the absorption of a solution can be evaluated. This is even more important when β-carotene solutions have been incubated, as increased absorption appears in the 300–400 mμ. region owing to the formation of *cis* isomers from all *trans*-β-carotene. When a photoelectric spectrophotometer is available the correction procedure of Morton & Stubbs (1946) allows the determination of the vitamin A contributed.

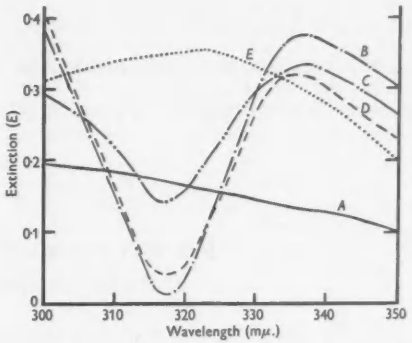

Fig. 2. The absorption spectra of β-carotene and mixtures of β-carotene and vitamin A in the spectral region 300–350 mμ.: *A*, pure β-carotene; *B*, β-carotene after incubation at 37° for 2·5 hr.; *C*, β-carotene after incubation at 37° for 2·5 hr. with iodinated casein; *D*, a computed curve for a mixture of vitamin A and incubated β-carotene, containing about 30 % of vitamin A; *E*, pure vitamin A.

The appearance of a carotene *cis* peak (Zechmeister, 1944) in the region of 330–335 mμ. after incubation could easily be mistaken for vitamin A absorption, especially when a spectroscope with a low dispersion is used. To illustrate this point a number of absorption curves are recorded in Fig. 2. The following points will be noted: (*a*) the reality of the *cis* peak of incubated carotene; (*b*) the difference between the curve of isomerized β-carotene and that of a mixture of this β-carotene and vitamin A (approx. 30 % vitamin A) is slight but well marked, and the presence of absorption due to the vitamin A in an incubated β-carotene solution could be detected even without application of the Morton & Stubbs (1946) correction if the curve were measured

photoelectrically; (c) that this difference might be missed using old (photographic) methods, for the changes, involving a shift of the band maximum only 1–2 mμ. and a small decrease in the slope on the short wave side of the maximum, are nearing the limits of detection; (d) that the curve of the β-carotene incubated alone is little different qualitatively from that obtained after incubation with iodinated casein.

Although experiments using more refined techniques have failed to substantiate earlier claims that thyroactive compounds catalyse the *in vitro* conversion of β-carotene into vitamin A, it is not to be assumed that the thyroid has no action on the *in vivo* metabolism and vitamin A. In fact, it has recently been shown in this laboratory that desiccated thyroid enhances and thiouracil reduces the absorption of β-carotene from the intestinal tract (Cama & Goodwin, 1949).

SUMMARY

1. It has not been possible to substantiate the claim that iodinated casein and desiccated thyroid are active in converting β-carotene into vitamin A *in vitro*.

2. The most likely error leading to this conclusion is the failure to distinguish the *cis* peak of isomerized β-carotene from the 326 mμ. absorption band of vitamin A.

We thank the Medical Research Council and the Ministry of Food for financial assistance, Prof. R. A. Morton for his interest and help, and Mr J. W. Lord (Messrs J. Bibby and Sons Ltd.) for the Russian translations.

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The Pyruvate-oxidase System in Brain and the Tricarboxylic Acid Cycle*

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The term pyruvate-oxidase system has been used in this laboratory to denote the complex of enzymes in brain which induce oxidation of added pyruvate. In earlier work (Long, Ochoa & Peters, 1939; Banga, Ochoa & Peters, 1939) the idea that this system in pigeon brain involved a tricarboxylic acid cycle (Krebs & Johnson, 1937; Krebs & Eggleston, 1940) was not supported. One of the main reasons was that, in contrast with kidney preparations, the increased oxygen uptake observed on addition of fumarate as well as pyruvate was not observed with citrate, which could not therefore be an intermediate; but, since then, the role of citrate has been changed to that of a side product rather than that of an intermediate (Wood, Werkman, Hemingway & Nier, 1941). It has always been difficult to account for the action of fumarate upon any other view than that of a tricarboxylic acid cycle, and we now think that the pyruvate-oxidase system in our homogenates does in fact include much of this cycle when fumarate is present; we are giving our evidence for this, though all details are not yet clear. The most important new point is that citrate and a substance, which is estimated as α-ketoglutarate, accumulate during the oxidation of pyruvate in the presence of fumarate and when malonate is also present.

* A preliminary account was given to the Biochemical Society on 22 January 1949 (Coxon, Liébecq & Peters, 1949).

† With the technical assistance of R. W. Wakelin.

METHODS

Estimations

Pyruvic acid. The Neuberg-Case method (Case, 1932) has been used as modified by Peters & Thompson (1934), the final colour of the hydrazone in ethanolic KOH being

measured in a Hilger absorptiometer with filter no. 7; the modified procedure described by Long (1942) has also been used. It has, however, been realized during this work that these methods give misleading results since they do not distinguish between pyruvate and α-ketoglutarate in a mixture, though the extinction of the latter is less for a given concentration.

The Friedemann & Haugen (1943) and the Straub (1936) methods have been employed as described by them, with the exception that in the case of the former a Beckman spectrophotometer was used for the final reading as explained below in connexion with α-ketoglutarate.

α-Ketoglutarate has been estimated most often by the method of Friedemann & Haugen (1943), employing the 420 and the 540 mμ. bands on the Beckman spectrophotometer instead of the simple photometer with different filters recommended by them. The enzymic method of Krebs (1938) was also used on a few occasions (as an independent check on the identity of the α-ketoglutarate). It is conceivable, however, as will be discussed later, that the substance which was determined by either method as α-ketoglutarate was originally present in the tissue suspension as oxalosuccinate and was converted to α-ketoglutarate by the subsequent analytical operations. Provided that the mixture contains more than 10% of α-ketoglutarate, the acids can be determined individually by the Friedemann & Haugen technique to an accuracy of ±5% in known mixtures. Duplicates agree to within ±2%.

Citric acid has been determined by the usual colorimetric method of Pucher, Sherman & Vickery (1936) and acetic acid by Long's (1938) modification of the method of Weil-Malherbe (1937). The details are given by Liébecq & Peters (1949). Latterly, preparatory to the citric acid estimation, all samples were boiled in presence of acid; the oxidation was carried out with permanganate, added drop by drop, and at 0·5N instead of 1·5N.

Preparations

Homogenates ('dispersions') from pigeon brain were made by grinding in a mortar according to the description of Banga *et al.* (1939); they were either dialysed as described by these authors or, more recently, some of the homogenates have been centrifuged for 30 min. in the cold room at 4000*g*, the centrifuged solid particles being then stirred into 1% KCl solution after separation from the supernatant fluid, to give a 'centrifuged homogenate'. It should be emphasized that the most satisfactory 'ground' homogenates are made by adding the cold KCl solution to the sticky pounded brain tissue drop by drop in the initial stages. Weights quoted in tables are of fresh tissue.

Adenosinetriphosphate (ATP) was prepared from the Ba salt obtained commercially (Boots Pure Drug Co. Ltd.) and was 80% pure.

The following acids were prepared by Mr R. W. Wakelin in this laboratory: α-ketoglutaric acid, m.p. 114·5 (corr.); *cis*-aconitic acid from *cis*-aconitic anhydride, m.p. 77°; *iso*-citric acid by hydrolysis of *iso*citric acid lactone, m.p. 138° after several recrystallizations from ethyl acetate; this last m.p. is lower than that given by Krebs & Eggleston (1944), but the compound behaved normally with aconitase.

RESULTS

It is convenient to give first the new facts and then to consider these in relation to previous work.

Accumulation of citrate

The experiments given in Table 1 are representative of several, and show that with dialysed

Table 1. *Citrate formed in absence and presence of fumarate by dialysed homogenate of pigeon brain*

(Period of incubation 30 min., following equilibration for 10 min. Temp. 38°. Tissue, approx. 200 mg.; fumarate, 0 or 10 μmol.; pyruvate, 30 μmol.; ATP, 1 μmol.; $MgCl_2$, 4 μmol. in 2·5 ml. in each bottle with 0·08M-phosphate buffer (pH 7·3). Addition of fumarate without pyruvate in control experiments produced somewhat less citrate than did pyruvate without fumarate.)

Exp. no.	Citrate formed (μmol./bottle): Without fumarate	With fumarate	Difference due to fumarate
1	0·36	2·43	+2·07
2	0·71	2·31	+1·60
3*	0·05	2·70	+2·65

* Centrifuged.

Table 2. *Effect of* cis-*aconitate and citrate upon oxygen uptake of centrifuged and dialysed brain dispersions*

(Tissue: approx. 200 mg./bottle; temp. 38°. Period of exp. 30 min. Each bottle contained 0 or 30 μmol. pyruvate; 0 or 10 μmol. fumarate; 1 μmol. ATP, 4 μmol. $MgCl_2$ and 0·08M-phosphate buffer pH 7·3 in 2·5 ml.)

			Oxygen uptake (μmol./bottle)									
			Fumarate absent						Fumarate present			
Exp. no.	Concentration (μmol./bottle): *cis*-Aconitate	Citrate	No other additions	*cis*-Aconitate	Citrate	Pyruvate	Pyruvate + *cis*-aconitate	Pyruvate + Citrate	No other additions	Pyruvate	Pyruvate + *cis*-aconitate	Pyruvate + citrate + *cis*-aconitate
1	45	0	—	—	—	5·0	6·7	—	2·5	9·8	—	—
2	40	9·6	—	—	—	—	—	—	3·0	12·8	13·0	10·0
3	15	11	—	—	—	—	—	—	—	12·7	—	12·5
4	20	20	2·8	2·5	2·3	4·8	5·6	4·5	3·9	7·3	—	—
5*	10	0	—	1·4	—	4·5	7·7	—	—	—	—	—
6*	10	0	—	2·3	—	4·2	8·0	—	—	8·9	—	—

* Dialysed and contained only 10 μmol. of pyruvate.

Table 3. *α-Ketoglutarate estimated as formed in absence and presence of fumarate in dialysed homogenates of pigeon brain*

(Tissue: approx. 200 mg./bottle; temp. 38°. Each bottle contained ATP, 1 μmol.; $MgCl_2$, 4 μmol. with 0·08M-phosphate buffer at pH 7·3; 0 or 10 μmol. fumarate, and pyruvate as indicated, in 3 ml.)

Exp. no.	Pyruvate added (μmol.)	Period of incubation (min.)	α-Ketoglutarate found (μmol./bottle): Fumarate absent	Fumarate present	Method of estimation
1	30	40	1	3·7	Friedemann & Haugen (1943)
2	18	40	Nil	2	Friedemann & Haugen (1943)
3	9	40	1	2·8	Friedemann & Haugen (1943)
4	30	10	Nil	2·5	Friedemann & Haugen (1943)
		40	Nil	3·5	Friedemann & Haugen (1943)
5	9	10	0·5	1·5	Friedemann & Haugen (1943)
		40	1	2·8	Friedemann & Haugen (1943)
6	9	40	2·4	4·5	Krebs (1938)
7	9	40	Nil	5·0	Krebs (1938)
8	0	40	Nil	0·35	Friedemann & Haugen (1943)
9	0	40	Nil	0·25	Friedemann & Haugen (1943)

brain homogenates respiring in pyruvate, citrate is formed, and that upon addition of fumarate much more citrate accumulates than in its absence. It was shown previously (Banga *et al.* 1939) that addition of citrate does not produce a 'fumarate' effect in these homogenates; hence any two-carbon fragment from pyruvate, after condensation with oxaloacetate and formation of citrate via *cis*-aconitate, cannot be oxidized further. Addition of *cis*-aconitate, together with pyruvate, gives some extra oxygen uptake in the absence of fumarate, tending to be less than that produced by fumarate; with fumarate and pyruvate there is no further increase. Citrate is inhibitory under some conditions (Table 2). Of other intermediates in the cycle, α-ketoglutarate is known from previous work in this laboratory to be oxidized by the brain preparation (see p. 323). The simplest interpretation of these facts is that in the brain homogenates some form of tricarboxylic acid cycle is operating; and that the oxidation sequence tends to be choked between *cis*-aconitate and α-ketoglutarate so leading to the accumulation of citrate.

Formation of α-ketoglutarate

During the respiration period of a brain homogenate, the concentration of a substance estimated as α-ketoglutarate is greatly increased if fumarate is present (Table 3). In the presence of pyruvate this can be demonstrated only by Friedemann's spectrophotometric method in the mixed 2:4-dinitrophenylhydrazones in alkaline solution. It cannot be demonstrated with the various modifications of the Neuberg-Case method (Case, 1932), though its presence can be deduced by estimating the total hydrazone in this way and subtracting the value for pyruvate obtained by the more specific procedure of Straub (1936). The enzymic method of Krebs (1938) provides an independent means of assay.

Acetic acid

The formation of acetate ion has not been so thoroughly investigated, but Table 4 shows that its behaviour in presence and absence of fumarate is the converse of that occurring with citrate and α-ketoglutarate. This indication that, in absence of fumarate, the pyruvate is largely degraded to acetate is consistent with the hypothesis advanced by Long & Peters (1939).

Table 4. *Formation of acetate in absence and presence of fumarate by dialysed homogenate of pigeon brain*

(Each bottle contained ATP, 1 μmol.; $MgCl_2$, 4μmol.; 0·08M-phosphate buffer (pH 7·3); pyruvate, 30μmol.; either 0 or 10 μmol. fumarate and tissue, approx. 200 mg. in 2·5 ml. Period of experiment: 30 min. + 10 min. equilibration.)

Exp. no.	Acetate found (μmol./bottle): Fumarate absent	Fumarate present
1	—	3·16
2	—	2·70
3	13·25	4·42
4	10·9	—

The effect of malonate and the origin of α-ketoglutarate

One of the important facts used in the proof of a tricarboxylic acid cycle in other tissues has been that there is formation of succinate from fumarate in the presence of excess of malonate (Krebs & Eggleston, 1940). Since excess of malonate inhibits the action of succinic dehydrogenase, it should also stop any possible formation of α-ketoglutarate from fumarate via succinate. That the α-ketoglutarate

Table 5. *Effect of malonate on oxygen uptake, disappearance of pyruvate and formation of citrate and α-ketoglutarate*

(Each vessel contained ATP, 1 μmol.; $MgCl_2$, 4 μmol.; 0·08M-phosphate buffer (pH 7·3); 10 μmol. pyruvate and dialysed brain homogenate equivalent to 150 mg. tissue. Malonate (where added), 0·025M. Time of incubation was 30 min. +10 min. equilibration. Temp. 38°. All figures given are in μmol./bottle, but the O_2 values have not been corrected for the equilibration period.)

	Exp. 1				Exp. 2		
	Pyruvate alone	With fumarate (10 μmol.)	With malonate	With fumarate +malonate	Alone	With fumarate (10μmol.)	With fumarate +malonate
Pyruvate disappearance	3·5	8·1	3·0	6·7	5·5	7·5	6·5
O_2 uptake	2·2	6·9	1·4	5·0	1·6	6·7	4·7
Citrate formation	—	2·5	0·15	2·4	—	—	—
α-Ketoglutarate formation	0·37	2·7	0·6	2·3	0·20	1·5	1·2

found in our experiments cannot be formed in this way is shown by Table 5; citrate and α-ketoglutarate are produced in presence of malonate.

It seems certain that the substance estimated in this work is α-ketoglutarate 2:4-dinitrophenylhydrazone. Not only does it behave spectrophotometrically in the same way as an authentic specimen, but also the substance can be converted into succinate and estimated enzymically as such. Nevertheless, there are some facts which make it uncertain at first sight whether this substance is present in the bottles as α-ketoglutarate; its amount is not appreciably increased by *iso*citrate (Table 6) and in

Table 6. *Comparison of α-ketoglutarate formation in presence of* cis*-aconitate and* iso*-citrate with that in presence of fumarate*

(Each bottle contained 10 μmol. pyruvate; 1·0 μmol. ATP; 4·0 μmol. $MgCl_2$ and 0·08M-phosphate buffer at pH 7·3. Final volume 3 ml./bottle. Temp. 38°.)

Exp. no.	*cis*-Aconitate added (μmol.)	*iso*Citrate added (racemic) (μmol.)	Fumarate added (μmol.)	α-Ketoglutarate found (μmol.)
1	10	0	0	0·75
	0	0	10	3·75
2	10	0	0	0·5
	0	0	10	2·5
3	0	20	0	0·1
	0	0	10	2·0
4	0	20	0	0·3
	0	0	10	2·0
5*	0	20	0	0·6
	0	0	10	1·8

* $MnSO_4$, 4 μmol. and coenzyme II, approx. 1 μmol. also added.

presence of malonate no extra α-ketoglutarate appears; α-ketoglutarate itself is known to be oxidized by the brain tissue. In view of this, we have considered the possibility that the 'α-ketoglutarate' is originally present as oxalosuccinate (Ochoa, 1948); however, both the statements of Ochoa and our observations suggest that this is so unstable that it would not long remain unchanged. The experiments with comparatively low concentrations of α-ketoglutarate given in Table 7 show

Table 7. *Fate of α-ketoglutarate added* in vitro

(Each bottle contained 1 μmol. ATP; 4 μmol. $MgCl_2$; 0·08M-phosphate buffer at pH 7·3, together with dialysed brain dispersion equivalent to 200 mg. tissue. Volume/bottle 2·5 ml. Preliminary equilibration for 10 min. Temp. 38°.)

Exp. no.	Malonate added (μmol.)	α-Ketoglutarate (μmol.) Initial	After 10 min.	After 20 min.
1	0	3·0	—	0·8
	0	6·0	—	1·7
	70	6·0	—	2·2
2	0	3·0	1·5	1·1
	0	6·0	2·9	2·1
	70	3·0	1·5	1·4

that, though larger amounts are readily oxidized, smaller amounts tend to remain as such and their rate of disappearance is not greatly influenced by malonate. Hence the behaviour resembles that in our experiments, and indicates that the α-ketoglutarate found can be simply that accumulating in the system during the oxidation.

Oxygen uptake and the balance sheet

In the past, it has been thought possible to draw up a balance sheet indicating that 1·5 mol. oxygen was used per mol. pyruvate (Banga *et al.* 1939), whilst the oxygen:pyruvate ratio for the extra metabolism produced by adenylic acid in the presence of fumarate has been found to approach more nearly the theoretical ratio for complete oxidation, viz. 2·5 (Long, 1946). In absence of adenylic acid or fumarate the ratio is considerably lower. Such calculations have been based on the assumption that the action of fumarate was a simple catalysis. Since we now consider that the fumarate enters into a complex series of oxidations such a balance sheet is unsatisfactory; less oxygen is used than would be necessary for complete oxidation of pyruvate and

we are forced to believe that incompletely oxidized products are left at various stages in the cycle. No balance sheet can be made until full information is available of the amounts present of all partially oxidized products.

DISCUSSION

Our experiments have led us to the belief that, as shown in other tissues, fumarate increases the oxygen uptake with pyruvate in brain because it supplies oxaloacetate, and that therefore the total pyruvate oxidase system in brain, as understood in this laboratory, includes some form of 'tricarboxylic acid' cycle. Though there are still discrepancies to be explained, these do not justify any longer a rejection of the idea of oxidation via a cycle, which is consistent with so much other evidence. At the same time, it is not yet clear just how citrate is formed and there are other incongruities which should be mentioned. Unlike kidney preparations, the brain homogenates do not oxidize citrate; they oxidize *cis*-aconitate only to a small extent. Further, as mentioned, added *cis*-aconitate gave rise to much smaller amounts of 'α-ketoglutarate' than did fumarate in a few experiments made by us (Table 6). We do not feel that the latter fact necessarily means that the α-ketoglutarate formed was arising by a separate path, because the conditions may be different when *cis*-aconitate is formed as an intermediate in presence of plentiful fumarate. The simplest view is that in the preparations used here the cycle is partially blocked between the stages *cis*-aconitate and α-ketoglutarate and also between α-ketoglutarate and succinate, but this interpretation still lacks the decisive proof that addition of some factor can remove the block.

Considering the oxidation in presence of fumarate, Banga *et al.* (1939) found that both adenine nucleotides and fumarate (as well as other acids in this series) were involved in the more complete oxidation of pyruvate. In further careful work, Long (1946) concluded that the same amount of pyruvate disappeared in presence and absence of fumarate, but that with fumarate, there was a more complete oxidation of the pyruvate disappearing. This led him to consider that the idea advanced by Banga *et al.* (1939), that fumarate increased the utilization of pyruvate, was erroneous, no allowance having been made for pyruvate formed during the experiment by oxidation of fumarate to pyruvate. It is shown here that part of the α-keto acid estimated by Neuberg-Case methods with fumarate present is α-ketoglutarate, which interferes only to an exceedingly small extent in the Straub (1936) method used by Banga *et al.* (1939). Hence the discrepancy might be explained by the failure of specificity in the Neuberg-Case methods. Nevertheless, the idea that the function of the fumarate is not simply catalytic is correct. Doubtless an exact knowledge of the amounts of various components of the cycle left in a partially oxidized state would lead to a quantitative balance sheet in these brain preparations, but, since so much will depend upon uncertain factors in the fragmentation, this investigation seems of secondary importance at the present time when various features of the cycle are more readily studied in other tissues (cf. Green, Loomis & Auerbach, 1948).

In absence of fumarate, the oxidation of pyruvate takes a different course and seems to go to acetate rather than *cis*-aconitate; as mentioned, this is consistent with the hypothesis of Long & Peters (1939), with the observations of Banga *et al.* (1939) in absence of fumarate, and with later work by Long (1943).

It is hoped to deal with this reaction later, and to leave to a later communication also the explanation of the apparent discrepancy between these results and some earlier reports from this laboratory based upon the assumption that succinic dehydrogenase and the pyruvate oxidase system were acting independently in brei. Some of the implications of these experiments for pharmacology have been discussed elsewhere (Peters, 1948).

SUMMARY

1. An accumulation of citrate and of α-ketoglutarate has been found to accompany the oxidation of pyruvate by dialysed 'dispersions' (i.e. finely ground homogenates) of pigeon brain in a medium containing fumarate and reinforced with magnesium ions and adenine nucleotide.

2. This evidence, despite the previous observation in this laboratory that citrate cannot replace fumarate in its effect on the oxygen uptake of these preparations, is taken to favour the operation in them of a tricarboxylic acid cycle when fumarate is present.

3. When fumarate is absent, the oxidation of pyruvate is associated with the formation of acetate.

We are grateful to Miss M. Pollock for help in some of these experiments.

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Acceleration of Bacterial Glutamic Decarboxylase and Glutaminase by Cetyltrimethylammonium Bromide

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It has been shown in a previous paper from this laboratory (Krebs, 1948) that cetyltrimethylammonium bromide ('cetavlon') can accelerate the decarboxylation of glutamate and the deamidation of glutamine by washed suspensions of *Clostridium welchii*. The present paper is concerned with the mechanism of this effect. As it is known that detergents may alter the permeability of cells (Gale & Taylor, 1947; Hotchkiss, 1946), it was thought that cetavlon might facilitate the entry of the substrates into the cells. To test this hypothesis, the effect of cetavlon on the rate of decarboxylation of glutamate and glutamine by cell-free extracts has been examined, but contrary to expectation the detergent gave the same effect as with intact cells. Thus the effect cannot be due to changes in the permeability of the cell wall.

METHODS

Organisms. *Clostridium welchii* S.R. 12 (National Collection of Type Cultures (N.C.T.C.) no. 6784) was grown for 16 hr. at 40° and a strain of *Escherichia coli* isolated locally and *Proteus morganii* (N.C.T.C. no. 2818) for 24 hr. at 30°, on the medium described by Krebs (1948). *Bacterium cadaveris* (N.C.T.C. no. 6788), *Cl. welchii*, (N.C.T.C. no. 6785), and *Streptococcus faecalis* (N.C.T.C. no. 6783), were grown as described by Gale (1947), but 'Pronutrin' (a commercial casein hydrolysate marketed by Herts Pharmaceuticals Ltd., Welwyn Garden City) was used instead of tryptic casein. *Clostridium septicum* (N.C.T.C. no. 547) served as a source of ornithine decarboxylase. The activity of the ornithine decarboxylase was increased 25-fold by reinforcing Gale's (1947) medium with extra ornithine (1 mg./ml. medium).

After growth, the cultures were centrifuged, the cells washed twice with 0·9% NaCl, and suspended in the appropriate buffer (Table 6). Phosphate buffer was used for the pH range from 7·0 to 5·5, acetate buffer for the range 5·5 to 4·0 and lactate buffer for the pH range from 4·0 to 2·5. The final concentration of the buffer was 0·10 M.

Chemicals. The preparations of L-glutamic acid, L-glutamine and cetavlon used in this work were those described by Krebs (1948). Ornithine was prepared from male herring roe, by the following method. The roe (250 g.) was heated on the water bath in a mixture of 150 ml. of conc. HCl and 50 ml. of $TiCl_2$ (20%). During the heating the roe was broken up with a glass rod and when fairly uniform it was autoclaved at 120° for 45 min. Water and HCl were removed by distillation *in vacuo* on a steam bath. The residual gum was dissolved in 100 ml. of hot water and 40% NaOH added until the pH was 6. The solution was filtered and, after adjustment of the pH to 7·6, an arginase preparation (15 ml. of 10% homogenized fresh rat liver) was added, and the mixture was incubated overnight. Urea was estimated manometrically and the ornithine content calculated from the urea formed.

Chemical determinations. The decarboxylation of glutamate and of glutamine was followed by measuring the evolution of CO_2 in a Warburg manometric apparatus. Buffer and substrate solutions were usually placed in the main compartment of the cup and the cells or extracts (see p. 326) containing the bacterial decarboxylase in the side arm. The gas space

was filled with N_2, and a stick of yellow phosphorus was put into the centre well to eliminate O_2. After 10 min. equilibration, the contents of the side arm and the main compartment were mixed. Readings were taken at 2 or 4 min. intervals. The rate of reaction is expressed as Q_{CO_2}, i.e. μl. CO_2/mg. dry wt. of cells/hr. In the case of extracts the Q_{CO_2} was calculated not from the dry wt. of extract used, but from the dry wt. of the cells from which the extract was made. The total volume of the fluid in each Warburg cup was 2·2 ml. and the amount of enzyme material added was generally equivalent to 2·0–5·0 mg. intact cells, varying with the activity of each batch of material, which was determined by a preliminary experiment. The concentration of enzyme material was such that not more than 50% of the added substrate was decomposed in the first 10 min., i.e. not more than 112 μl. CO_2 was produced. Within a suitable range the rate of decarboxylation of glutamine in extracts was proportional to the enzyme concentration. Under the conditions used the range was between 1 and 20 mg./cup in the absence of cetavlon and 1 and 10 mg. in its presence. At higher concentrations the rate was too rapid for precise measurement. At lower concentrations the rate fell off after about 5 min., possibly owing to the destruction of the enzyme in dilute solution. Under these conditions the rate was approximately linear for at least 10 min. and the Q_{CO_2} was calculated from the linear rate of gas evolution. Any deviation from this procedure is mentioned later. Ammonia was determined by the method of Parnas & Heller (see Parnas, 1934).

Preparation of cell-free extracts. The washed cells were broken by grinding with powdered pyrex glass (Wiggert, Silverman, Utter & Werkman, 1940; McIlwain, Roper & Hughes, 1948). The degree of cell disintegration was checked by microscopic examination of a Gram-stained smear of the ground mixture diluted with water. Usually no intact cells were found, and the cell debris of *Cl. welchii* was Gram-negative. The clay-like mass of broken cells and glass was mixed with 0·05-M acetate buffer, pH 4·6, in the proportion of 2 ml. of buffer for 1 g. of wet cells, and centrifuged for 10 min. at about 4000 rev./min. The supernatant was poured off and the residue extracted twice more with the same amount of buffer. The three extracts were combined, centrifuged again, and the slightly opalescent solution stored at about 2° in a stoppered cylinder.

Extracts of *Cl. welchii* contained about 80% of the original glutamic decarboxylase activity (see Table 1). Extracts of equal potency were obtained when the above buffer was replaced by other solutions (0·9% NaCl, 0·05 M-acetate buffer pH 5·0 or 6·0, or 0·05 M-phosphate buffer pH 7·0). The yield of glutaminase was difficult to measure with any certainty owing to the change in behaviour of the enzyme after extraction. For instance, when the rate of decarboxylation of glutamine was measured at pH 4·9 the yield of glutaminase appeared to be 50–60% without cetavlon and 80–90% when it was added. When the activity was measured at pH 4·1 the yield of glutaminase appeared to be negligible in the absence of cetavlon, but when the detergent was added the activity was often greater than that of the intact cells (see Table 1).

Extracts of *Proteus morganii* decarboxylated both substrates more slowly than the intact cells both in the absence and presence of cetavlon.

Esch. coli gave extracts which under the conditions used appeared to contain 25–40% of the original decarboxylase and 50–100% of the original glutaminase activity.

Stability of the enzyme preparations. As already reported (Krebs, 1948), the decarboxylase and glutaminase in intact cells of *Cl. welchii* suspended in 0·05 M-acetate buffer, pH 4·5, and stored at about 2° keep for several weeks. The glutamic decarboxylase activity of an extract from *Cl. welchii* showed no decrease after 9 days at 2°, but a decrease of 35% after 30 days at 2°. In the same sample, the ability to decarboxylate glutamine fell off more rapidly; 30% of the original activity was lost in 9 days and 55% after 30 days at 2°. In another experiment, in which an extract was incubated without substrate for 20 min. at 40° in 0·1 M-acetate buffer pH 4·6, 32% of the glutamic decarboxylase and 80% of the glutaminase activity disappeared. Thus the glutaminase in the extracts is less stable than the glutamic decarboxylase. Most of the work described in this paper was carried out with glutamic acid as the substrate in order to avoid complications arising from instability of the glutaminase.

RESULTS

The effects of cetavlon on the rate of decarboxylation of glutamate and glutamine in intact cells and extracts. Table 1 shows that cetavlon accelerates the decarboxylation of glutamate and glutamine in extracts and in washed cells of *Cl. welchii*, *Proteus morganii* and *Esch. coli*.

Table 1. *The effect of cetavlon on the rate of decarboxylation of glutamate and glutamine by intact cells and extracts*

(Temp. 40°; substrate, 0·0045 M; cetavlon, 0·0025 M; 0·1 M-acetate buffer pH 4·6.)

Substrate added	Q_{CO_2} Intact cells	Q_{CO_2} Extract
Cl. welchii		
Glutamate	270	224
Glutamate and cetavlon	535	405
Glutamine	114	5
Glutamine and cetavlon	270	290
Proteus morganii		
Glutamate	40	44
Glutamate and cetavlon	310	82
Glutamine	45	23
Glutamine and cetavlon	250	56
Esch. coli		
Glutamate	102	11
Glutamate and cetavlon	425	160
Glutamine	89	40
Glutamine and cetavlon	270	270

With intact cells of *Cl. welchii* at pH 4·1 the degree of acceleration of the decarboxylation of glutamate on the addition of cetavlon was from two- to three-fold. In extracts the effect of cetavlon was slightly less than with the intact cells, but of the same order. On the other hand, the decarboxylation of glutamine showed striking differences between intact cells and extracts of *Cl. welchii* (see Table 1). In the absence of cetavlon the extracts were 20% less active than the intact cells even at pH 4·9, which has been shown to be the optimum pH for the decarboxylation of glutamine (Krebs, 1948). The low activity of the

extracts towards glutamine is not due to an irreversible destruction of the enzymes because the addition of cetavlon greatly increases the activity, in some cases to a Q_{CO_2} level higher than that of the intact cells.

In the case of *Proteus morganii* the acceleration by cetavlon was more marked in intact cells than in extracts. It will be shown later for *Cl. welchii* that the pH and the substrate concentration can greatly influence the effect of cetavlon. The role of these factors has not been investigated for *Proteus morganii*.

In *Esch. coli* the effects of cetavlon were very pronounced both on intact cells and on extracts, irrespective of whether glutamate or glutamine was the substrate.

The effect of cetavlon on the pH *curve of the glutamic decarboxylase in extracts of* Clostridium welchii. As shown in Fig. 1 addition of the detergent to extracts

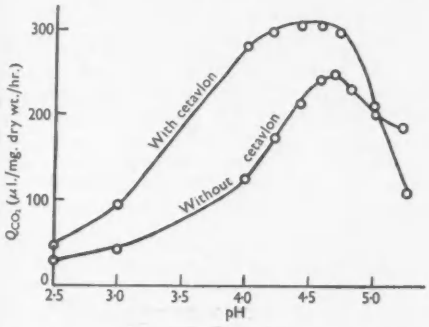

Fig. 1. The effect of cetavlon on the pH curve of the extracted decarboxylase of *Cl. welchii*. 0·1 M-lactate, pH 2·5–3·0; 0·1 M-acetate, pH 4·0–5·0; 0·0025 M-cetavlon if added; substrate 0·0045 M-glutamate; extract corresponding to 2·0 mg. dry wt. of cells; total volume 2·2 ml.; temp. 40°.

of glutamic decarboxylase from *Cl. welchii* changed the shape of the pH curve of the enzyme by broadening the optimum towards the lower pH range. This effect is similar to that found with intact cells (Krebs, 1948). Above pH 4·8 inhibitions varying from 5 to 20 % were found on the addition of cetavlon to both extracts and cells. As the degree of acceleration by cetavlon was greatest between pH 4·0 and 4·2 its effect has been measured at pH 4·1.

It is noteworthy that the pH optimum in the absence of cetavlon is 4·6 with extracts and 4·1 with cells. Similar changes in the optimum pH of the tyrosine decarboxylase on extraction from *Strep. faecalis* have been described by Epps (1944). Another point of interest is that the extracted enzyme has a sharper pH optimum than has the enzyme of the intact cells.

The effect of cetavlon on the stability of glutamic decarboxylase in extracts of Clostridium welchii. There was loss of activity of the decarboxylase without the addition of cetavlon when the extract was incubated at 40° at pH 4·9, 4·1, 3·0 and 2·5 (Table 2). Cetavlon had little effect on this loss of

Table 2. *The effects of cetavlon on the stability of the glutamic decarboxylase in extracts of* Clostridium welchii

(Main compartment: 1·0 ml. 0·2 M-buffer, 0·5 ml. extract, equivalent to 2·0 mg. dry wt. cells; if added, 0·1 ml. 2 % cetavlon. Side arm: 0·5 ml. 0·01 M-glutamate. Total volume 2·2 ml. Contents mixed after 10 min. or 20 min. at 40°.)

	Q_{CO_2} at 10 min.		Q_{CO_2} at 20 min.	
Buffer	Without cetavlon	With cetavlon	Without cetavlon	With cetavlon
Acetate pH 4·9	264	264	180	180
Acetate pH 4·1	142	250	82	160
Lactate pH 3·0	52	190	26	124
Lactate pH 2·5	33	99	10	43

activity. The accelerating effect of cetavlon after partial destruction of the decarboxylase was about the same as before incubation. The detergent therefore neither destroys the activity of the enzyme nor protects it from destruction.

The effect of cetavlon on the solubility of the extracted decarboxylase of Clostridium welchii. On adjusting the pH of extracts to below pH 4·3 with acetate buffer a precipitate was formed. About 60 % of the decarboxylase activity was precipitated at pH 4·1, and both the precipitate and the mother liquor gave the cetavlon effect (Table 3, Exp. 1). In the presence of cetavlon almost all the decarboxylase was in the precipitate (Table 3, Exp. 2), and the activity of the washed precipitate was not increased by the addition of cetavlon. At pH 4·6, the addition of cetavlon produced a precipitate, which, even after washing, contained decarboxylase whose activity was again unaffected by the addition of further cetavlon. About 70 % of the activity was recovered in the precipitates formed when cetavlon was present, but quantitative assay was difficult as the precipitates were very sticky and adhered to pipettes and test tubes. The material was insoluble in acid and neutral buffers and lost activity if the pH was above 7·0. On the other hand, precipitates obtained by adjusting the pH of the extract to pH 4·1 without the addition of cetavlon redissolved at pH 6 and were stable even at pH 9. Above pH 9 the activity was lost rapidly.

If the extract was adjusted to pH 4·1 in the presence of methyl orange the precipitate was stained orange when cetavlon was absent, and this orange colour was removed slowly by washing. When cetavlon was present the precipitate was bright yellow and the colour was not removed after four

Table 3. *The effect of* pH *and cetavlon on the precipitation of the glutamic decarboxylase*

(Each fraction was transferred in 0·5 ml. water to the side arm; 0·0045 M-glutamate, 0·1 M-acetate buffer pH 4·1 and cetavlon, if added, 0·0025 M, were in the main compartment. Total volume 2·2 ml.)

Condition of precipitate	Fraction tested	Q_{CO_2} Without cetavlon	Q_{CO_2} With cetavlon
Control. Original extract not precipitated		108	344
Exp. 1. Extract taken to pH 4·1 with acetate; left 15 min. at 2°; centrifuged; precipitate washed once with 0·05 M-acetate buffer pH 4·1	Supernatant	59	118
	Precipitate	88	188
Exp. 2. Extract precipitated as above but in the presence of $2·5 \times 10^{-3}$ M cetavlon; precipitate washed once with 0·005 M-acetate buffer pH 4·1	Supernatant	0	0
	Precipitate	258	258
Exp. 3. Extract adjusted to pH 4·6, diluted to the same volume as the above experiments and cetavlon added (final concentration $2·5 \times 10^{-3}$M). Precipitate washed as above	Supernatant	0	0
	Precipitate	262	262

washings with 0·05 M-acetate buffer pH 4·6 or 4·1, or with 0·05 M-phosphate buffer, pH 7·0. Hartley (1923) has shown that some cationic detergents change methyl orange to a bright yellow colour. This colour change is not due to any change in pH and is connected with micelle formation by the detergents. The fact that methyl orange becomes yellow when absorbed on to precipitated enzyme material indicates that detergent-protein complexes have been formed (Putman & Neurath, 1945; Lundgreen, 1945).

Microscopic examination showed that the precipitate obtained by acidifying the extract to pH 4·1 was composed of very fine granules when cetavlon was absent, and coarse granules when the detergent was present. When these precipitates were shaken with glutamate at 40° the one without cetavlon did not change in appearance, whilst the one with cetavlon became coarser. This effect is similar to that found by Klarman & Wright (1946) on the clumping of bacteria by detergents. Thus cetavlon neither solubilizes nor peptizes the enzyme-containing material, but precipitates it and forms complexes.

The effect of cetavlon on the rate of decarboxylation at varying concentrations of glutamate. At 30° the rate of decarboxylation of glutamate (2 mg. dry cells in 2·2 ml.; substrate concentrations 0·0023–0·046M) was found to be steadier than at 40°, and the following experiments were therefore carried out at 30°. Table 4 shows the effect of cetavlon on the rate of decarboxylation at different glutamate concentrations by washed cells and extracts. It will be seen that the effect of the detergent was greatest at the lowest substrate concentrations, whilst at high substrate concentrations when the enzyme is almost saturated with substrate it has little or no effect. The increase being, in the case of the cells, 147% at 0·0023 M-glutamate and only 13% at 0·046 M-glutamate. These results can be explained on the assumption that cetavlon increases the apparent affinity of the substrate for the enzyme. The concentrations of substrate giving half the maximum velocity, determined graphically from the results given in Table 4, are as follows: intact washed cells, without cetavlon, 0·009M; with cetavlon, 0·004M. Extract of cells, without cetavlon, 0·0095M; with cetavlon, 0·0045M.

Table 4. *Effect of cetavlon on the rate of decarboxylation at varying concentrations of glutamate*

(In each Warburg cup, 0·1 M-acetate buffer (pH 4·1), glutamate solution, 0·0025 M cetavlon, if added; enzyme in the side arm; total volume 2·2 ml.; temp. 30°.)

Glutamate (M)	Q_{CO_2} Without cetavlon	Q_{CO_2} With cetavlon
Intact washed cells		
0·046	890	1003
0·023	790	990
0·011	535	745
0·0046	268	455
0·0023	109	268
Extract of the above cells		
0·046	535	675
0·023	492	582
0·011	330	465
0·0046	211	362
0·0023	125	231

Assuming that the half-maximum velocity is a measure of the apparent affinity of the enzyme for the substrate, the addition of cetavlon increased the affinity of the decarboxylase about twofold, both with intact cells and with extracts.

Changes in the apparent affinity of enzyme for substrate can be brought about by competitive inhibitors, addition of a competitive inhibitor decreasing, and its removal increasing, the apparent affinity. The effect of cetavlon could thus be explained by the assumption that it removes a competitive inhibitor which accompanies the bacterial decarboxylase and glutaminase. This conception is supported by the graph shown in Fig. 2, in which a plot is made of the reciprocal of the velocity $1/v$ against the reciprocal of the substrate concentration $1/S$ (Lineweaver & Burk, 1934). The two lines referring to extracts show the same ordinate inter-

cept, i.e. the same maximum velocity, but the addition of cetavlon has decreased the slope. According to Lineweaver & Burk (1934) this is expected if a competitive inhibitor is removed.

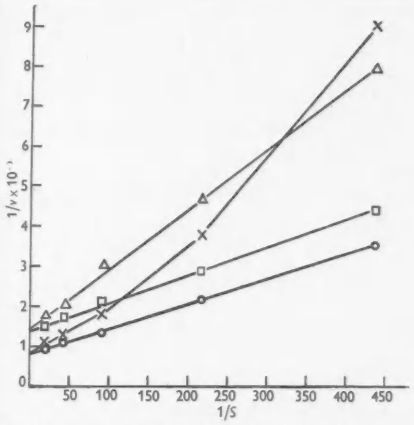

Fig. 2. Data in Table 4 plotted as suggested by Lineweaver & Burk (1934); v=reaction velocity, expressed as Q_{CO_2}; S=substrate concentration (M). ×—×, intact cells without cetavlon; ○—○, intact cells with cetavlon; △—△, extract without cetavlon; □—□, extract with cetavlon.

The plot of the data obtained with intact cells, when cetavlon is absent and the substrate concentration is low, does not give a straight line. This suggests that under these conditions the rate of entry of glutamate into the cell limits the rate of decarboxylation. This conclusion is further supported by the fact that a straight line is obtained from the data with extracts.

The plot obtained from the results with intact cells in the presence of cetavlon shows two points of interest. First, the line is straight, suggesting that cetavlon eliminates diffusion as a controlling factor. Secondly, the line is almost parallel to that showing the effect of cetavlon in the extract. This means that although cetavlon shows an effect on the rate of entry of glutamate into the cell, the apparent affinity of the enzyme in intact cells is also increased, in the same way as in extracts. The fact that the acceleration by cetavlon in intact cells of *Cl. welchii* is slightly higher than that found in extracts (see Table 1) can probably be explained by an additional effect of the detergent on the permeability of the cell.

The effect of cetavlon on the rate of decarboxylation of glutamine at varying substrate concentrations. The rates of decarboxylation at varying concentrations of glutamine by extracts of *Cl. welchii* are shown in Table 5. A concentration of substrate sufficient to saturate the enzyme was not reached, as the solubility of glutamine at 30° is approximately 0·23 M. The decarboxylation is extremely slow at low concentrations of substrate without cetavlon and is increased by the addition of cetavlon, the cetavlon effect decreasing as the substrate concentration is raised. This suggests that the apparent affinity of the glutaminase is increased in a manner similar to that of the decarboxylase.

Table 5. *The effect of cetavlon on the rate of decarboxylation of varying concentrations of glutamine by extracts of* Clostridium welchii

(0·1 M-acetate buffer pH 4·1; glutamine and 0·0025 M-cetavlon in the centre; enzyme in the side arm; total volume 2·2 ml.; temp. 40°.)

Glutamine (M)	Q_{CO_2} With cetavlon	Q_{CO_2} Without cetavlon
0·23	1160	440
0·12	850	250
0·046	490	143
0·023	330	78
0·0124	260	24
0·0046	215	1·0–5·0
0·0023	144	1·0–5·0

Treatment of the results, according to Lineweaver & Burk (1934), does not give as clear a result as in the case of the decarboxylation of glutamate. Whilst the plot of $1/S$ against $1/v$ is a straight line for extract with cetavlon, that for the extract alone gives an S-shaped curve. If the results for extract alone are plotted in the form $1/v$ against $1/S^2$ the line is straight. With a single enzyme this result suggests that either a ternary collision was involved or that an inhibitor was present.

The effect of cetavlon on other enzyme systems. No acceleration of any other enzyme system has been found. The systems tested and the methods of testing are summarized in Table 6.

DISCUSSION

The acceleration by cetavlon of the decarboxylation of glutamate and glutamine, which had previously been reported with intact cells (Krebs, 1948), has now been shown to take place with extracts of *Cl. welchii*, *Proteus morganii* and *Esch. coli*. The effects are generally of the same order as with intact cells; thus the main action cannot be due to an effect on permeability of the cell walls. At low substrate concentrations the degree of acceleration with intact cells was slightly higher than with extracts, and this may be due under these conditions to an additional effect of cetavlon on cell permeability.

Examination of the cetavlon effect at varying substrate concentrations showed that the detergent increased the apparent affinity of the decarboxylase for glutamate. Changes in the affinity of an enzyme for its substrate can be brought about by the

Table 6. *The effect of cetavlon on the reaction rates of enzymes other than glutamic decarboxylase and glutaminase of* Clostridium welchii

Name of enzyme	Source of enzyme	Method of preparation and experimental conditions	pH at which the system was tested	Effect of cetavlon (0·0025 M and 0·01 M final concentration)
Ornithine decarboxylase	Washed intact cells of *Cl. septicum*	Gale (1947)	5·5, 5·0, 4·0	None
Tyrosine decarboxylase	Washed intact cells of *Strep. faecalis*	Gale (1947)	5·5, 5·0, 4·0	None
Tyrosine decarboxylase	Extract of acetone dried cells of *Strep. faecalis*	Epps (1944)	5·5, 5·0, 4·0	None
Arginine decarboxylase	Washed intact cells of *Esch. coli*	Gale (1947)	5·0, 4·0	None
Arginine decarboxylase	Extract of *Esch. coli* disintegrated by grinding glass	Tested as with intact cells	5·0, 4·0	None
Histidine decarboxylase	Washed intact cells of *Cl. welchii* strain (6785)	Gale (1947)	5·0, 4·0, 3·0	None
Lysine decarboxylase	Washed intact cells of *Bact. cadaverie*	Gale (1947)	5·0, 4·0	None
Glutamic decarboxylase	Extracts of carrots and squash	Schales, Mims & Schales (1946)	5·0, 4·0, 3·0	None
Urease	Extract of Jack-bean meal		5·0, 4·0	None

following mechanisms: (*a*) increased concentration of substrate in the vicinity of the enzyme (Van Slyke, 1942); (*b*) an alteration in the properties of the enzyme by reaction with an added substance; (*c*) removal of an inhibitor.

It seems unlikely that mechanism (*a*) is involved, as transport of material by surface-active agents has been demonstrated only in the case of lipophilic substances (Dean & Vinograd, 1942), and neither glutamate nor glutamine is lipophilic.

The reaction of surface-active agents with enzymes usually leads to inactivation of the enzyme (Putman, 1948; Valko, 1946). On the other hand, Bodin & Hill (1945) found that the addition of anionic detergents to prototyrosinase increases the activity; they suggested that the increase was due to formation of enzyme material from a precursor, but did not investigate the effect of detergents on the affinity of the enzyme.

The data on the effect of cetavlon at varying substrate concentrations strongly suggest that a competitive inhibitor of the glutamic decarboxylase is removed by cetavlon, either by solubilization of the inhibitor on micelles (for this effect see Alexander & Trim, 1946), or by combination with the inhibitor to form a complex.

The experiments on the effect of cetavlon on the apparent affinity of the glutaminase do not give such a clear picture as those on the glutamic decarboxylase. However, the results suggest that cetavlon accelerates the glutaminase by the removal of an inhibitor.

SUMMARY

1. Cell-free extracts containing glutamic decarboxylase and glutaminase have been prepared from *Clostridium welchii*, *Proteus morganii* and *Escherichia coli* by grinding the cells with powdered glass, adding buffers to the ground mass and centrifuging.

2. Of the original decarboxylase of the intact cells, about 60% was recovered in extracts from *Cl. welchii*, 30–40% from *Proteus morganii* and 25–40% from *Esch. coli*. The glutaminase activity of extracts was between 50 and 100% of that of the intact cells.

3. The activity both of the decarboxylase and of the glutaminase in the extracts was increased by the addition of cetavlon.

4. The acceleration of the decarboxylase by cetavlon in intact cells and extracts of *Cl. welchii* became smaller as the substrate concentration was raised, i.e. the apparent affinity of the decarboxylase for glutamate seems to be increased by the addition of cetavlon.

5. Bacterial decarboxylases acting on ornithine, tyrosine, arginine, lysine and histidine are not accelerated by the addition of cetavlon, nor is the glutamic decarboxylase of *Daucus carota* (carrot) or *Cucurbita pepo* (squash).

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Factors Influencing the Polysaccharide Content of *Escherichia coli*

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It is known that bacteria build up reserves of polysaccharides within the cell, but little work has been done on the factors which influence their accumulation. Our object was to investigate factors influencing polysaccharide storage and the role which storage plays in the metabolism of the bacterial cell. Dawson & Happold (1943) reported that D-phenylalanine caused increased storage of polysaccharide when *Escherichia coli* was grown on a glucose, but not on a mannose, medium. Gale (1947) has stated that polysaccharide formation occurs within the cell when *Esch. coli* is allowed to metabolize glucose in excess, but this material is itself metabolized as soon as the external glucose is exhausted. To investigate this, storage was examined where onset of the stationary phase was determined by both glucose and nitrogen exhaustion in the medium.

Additional interest in variations of polysaccharide content was occasioned by frequent observations that a fall in bacterial mass, as indicated by turbidity measurements, occurred in the early stationary phase. Monod (1942) also observed decrease in turbidity, attributed to a decrease in cell size, in this phase, and it was of interest to see whether this was related to polysaccharide content.

EXPERIMENTAL

The organism used throughout this work, unless otherwise stated, was no. 5928 of the National Collection of Type Cultures.

Growth cycles were followed turbidimetrically, using a Spekker photoelectric absorptiometer with neutral filters, H 508, and microcells. Since we have shown that values for the polysaccharide content are dependent on the stage reached in the stationary phase we have been careful to ensure that samples were taken at the same phase of growth. In every experiment the inoculum has been taken from a medium containing the same constituents as the medium to be used in the experiment, and from the early stationary phase, under which conditions minimum lag is obtained (Hinshelwood & Lodge, 1943).

Flasks were incubated in a thermostat at 38°. Determinations of pH were made with a Marconi type TF 717A pH meter. Dry-weight determinations were made on 1 ml. bacterial suspension placed on a watch glass and heated overnight in an electric oven (80–90°) to constant weight.

For polysaccharide determinations portions of at least 250 ml. culture were centrifuged and the supernatant reserved for pH and sugar determinations. The cells were twice suspended in distilled water and again centrifuged, taken up in 10 ml. distilled water, filtered through glass wool and used for polysaccharide and dry-weight determinations. No sugar was detected in the supernatant after final centrifugation.

Media

Medium A, a simple salt medium containing: 5·4 g. KH_2PO_4, 1·2 g. $(NH_4)_2SO_4$, 6·6 g. glucose and 0·66 g. $MgSO_4.7H_2O$/l.; pH adjusted to 7·12 with NaOH.

Medium B, a complex amino-acid medium containing: 0·1 g. L-cystine, 0·5 g. glycine, 1 g. DL-valine, 5 g. sodium glutamate, 1·5 g. Na lactate, 5 g. NaCl, 2·5 g. $Na_2HPO_4.12H_2O$, 0·35 g. KH_2PO_4, 0·30 g. $MgSO_4.7H_2O$, 3·5 mg. $FeSO_4.7H_2O$/l.; pH adjusted to 7·5 with NaOH.

Addition of sugar was made separately. The medium and sugar were sterilized separately by boiling on successive days.

Determination of bacterial polysaccharide

The polysaccharide content of *Esch. coli* has been determined by Dawson & Happold (1943), using the Sahyun (1931) method for the determination of tissue glycogen. Since this involves treatment with 10 N-KOH in a boiling water bath the method appeared open to objection on the grounds that this would result in loss of such constituents as amino sugars. Furthermore, adsorption on charcoal is also employed and this may result in low recoveries. The determination of reducing sugar by the Hagedorn & Jensen (1923) technique, which is not specific for reducing sugar, is also open to objection. These possibilities were, therefore, investigated.

To determine reducing sugar the method of Somogyi(1937) was adopted. This reagent was found to keep well, and periodic restandardization revealed no significant change in reduction equivalent. The hydrolysis of bacterial polysaccharide was effected with H_2SO_4 and optimum conditions for this, and the effect of alkali treatment, were investigated.

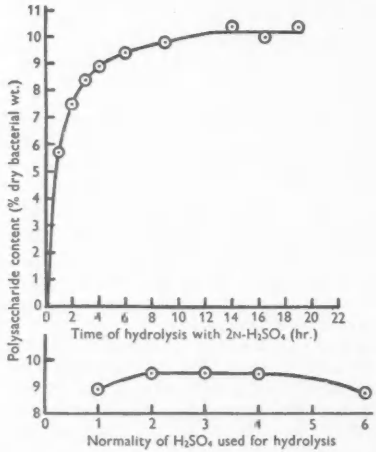

Fig. 1. Polysaccharide content of *Esch. coli* determined by the Somogyi (1937) method, after varying periods of hydrolysis of washed cell suspensions with 2N-H_2SO_4, and after 16 hr. hydrolysis of washed cell suspensions with varying concentrations of H_2SO_4.

Effect of length of hydrolysis on polysaccharide determination. Portions (5 ml.) of a bacterial suspension were hydrolysed in sealed ampoules with 2N-H_2SO_4 for varying periods in a boiling water bath, the contents transferred quantitatively, neutralized and made to 50 ml. Portions (5 ml.) were used for reducing sugar determinations (triplicate). The polysaccharide content of the organism is expressed in terms of glucose equivalents as a percentage of the dry bacterial weight. Fig. 1 shows that hydrolysis is complete at approximately 10 hr. Accordingly, for convenience, all hydrolyses were carried out overnight.

Effect of acid strength on polysaccharide determination. Fig. 1 shows the variation in polysaccharide content obtained when hydrolysis of the bacterial suspension was carried out with various acid strengths. It will be seen that there is little difference over the range 1–6 N, maximum effect being obtained with 2–4 N-acid. Accordingly, we decided upon 2 N-acid for hydrolysis in all subsequent work.

Effect of pre-treatment with alkali. The effect of treatment with 2 N-NaOH prior to acid hydrolysis was investigated. Portions (5 ml.) of bacterial suspensions were made 2 N with respect to NaOH, and the unsealed ampoules covered with glass bulbs and placed in the boiling water bath for given periods. They were then removed, made 2 N with respect to H_2SO_4, sealed and returned to the water bath. Treatment with 2 N-NaOH for 2 hr. prior to 10 hr. hydrolysis with 2 N-H_2SO_4 gave a polysaccharide content of 7·1% dry bacterial weight as compared with 15·5% with the control. Pre-treatment with NaOH therefore results in a much lower figure for polysaccharide content.

Comparison of polysaccharide determinations by the Somogyi and Hagedorn & Jensen methods. For convenience, hydrolysis in this experiment was conducted for 2 hr. only, and then the reducing sugar liberated determined by both the Somogyi (1937) and Hagedorn & Jensen (1923) methods. In three different experiments the latter method gave much higher figures, in some cases double the Somogyi value: viz. Somogyi 3·5, 2·7, 3·7 and Hagedorn & Jensen 7·3, 5·3 and 5·6% dry bacterial weight respectively. Clearly, the Hagedorn & Jensen method is determining reducing substances other than sugars. The findings that alkali causes destruction of approximately 50% bacterial polysaccharide, and that the Hagedorn & Jensen method of reducing sugar determination gives very high results with bacterial hydrolysates, was fully borne out when a direct comparison of Dawson & Happold's (1943) method was made with our own (see p. 333).

Comparison of polysaccharide determinations by the Dawson & Happold technique and by acid hydrolysis. Seeking to explain the difference in observations recorded by Dawson & Happold (1943) using the Sahyun (1931) method, and ourselves, employing acid hydrolysis, on the effect of D-phenylalanine, we undertook a direct comparison of both methods and also carried out the reducing sugar determinations by the Hagedorn & Jensen and the Somogyi techniques. Thus a comparison was afforded of both important stages in the procedure: first hydrolysis and then reducing sugar determination.

Two flasks were set up each containing 1 l. of medium *B* to which 1% (w/v) of glucose and DL-tryptophan was added. D-Phenylalanine (0·13 g.) was added to one of these flasks and both inoculated with two loops of bacterial suspension. Polysaccharide determinations were carried out, when the stationary phase had been reached, by both methods. The results (Table 1) confirm our previous findings, namely, that destruction of 40–50% of bacterial polysaccharide is brought about by the KOH treatment in the Sahyun method, and also that the Hagedorn & Jensen method of sugar determination gives results which are about 50% too high, presumably due to the presence of reducing substances other than sugars. Quite clearly the Sahyun method for glycogen is not suitable for total bacterial polysaccharide determinations, and neither is the Hagedorn & Jensen method suitable for the determination of reducing sugar liberated from such material. All our work therefore has, with justification, been based on acid hydrolysis of the cells followed by determination of reducing sugar by the Somogyi method.

It will be noted that, using the Sahyun and Hagedorn & Jensen methods, the polysaccharide content is slightly higher in the presence of D-phenylalanine. However, using the method of Dawson & Happold, the polysaccharide content obtained is very much greater than that recorded by them (about 5%) and this is apparently due to a strain difference. To investigate this possibility the polysaccharide content of various strains has been examined (see p. 335).

Table 1. *Comparison of polysaccharide determinations by the Sahyun and acid-hydrolysis methods with reducing sugar determinations by the Hagedorn & Jensen and Somogyi techniques*

	Polysaccharide content (% dry bacterial wt.)			
Method of hydrolysis	Sahyun		2 N-H_2SO_4	
Method of reducing-sugar determination	Hagedorn & Jensen	Somogyi	Hagedorn & Jensen	Somogyi
Control	15·5	10·8	28·2	19·5
0·013% (w/v) D-phenylalanine	17·5	12·8	26·8	18·7

Accuracy of the method. Two sets of duplicate determinations were carried out, with different dry weights; hydrolyses and reducing sugar determinations being all carried out independently, all possible experimental variables were introduced. For two different samples of *Esch. coli* grown under different conditions the polysaccharide contents (% dry bacterial weight) were: sample 1—13·5, 13·3; sample 2—17·5, 17·4.

Final method for bacterial polysaccharide determinations. Portions (5 ml.) of bacterial suspension (washed free from sugar present in medium as previously described) were placed in ampoules, 0·3 ml. conc. H_2SO_4 added to each to make 2 N with respect to acid, and the ampoules sealed and placed in a boiling water bath overnight. They were then opened and the contents transferred quantitatively, with filtering to remove debris, to 50 ml. graduated flasks, neutralized and made to the mark with water. Portions (5 ml.) were used in triplicate for the determinations with 5 ml. Somogyi reagent. Liberated I_2 was titrated with 0·005 N-$Na_2S_2O_3$, which was made from 0·1 N-stock solution immediately before use.

RESULTS

The variation of polysaccharide content of Escherichia coli *at various phases of the growth cycle*

Variations in polysaccharide content with time. Growth was followed by turbidity measurements in a medium in which glucose was known to be in excess (simple medium *A*). The fluctuation of polysaccharide content with time is shown in Fig. 2. It is seen that between 20 and 38 hr. a fall in polysaccharide occurs. In another experiment the polysaccharide content during this phase of growth was followed more fully over a period of 46 hr. The polysaccharide content 1·75 hr. before onset of the stationary phase was 16·9%; 22 hr. after onset it was 12·2% and 42 hr. after, 9·0%. A fall in polysaccharide content over long periods of the stationary phase has been confirmed in several subsequent experiments concerned with the influence of other factors.

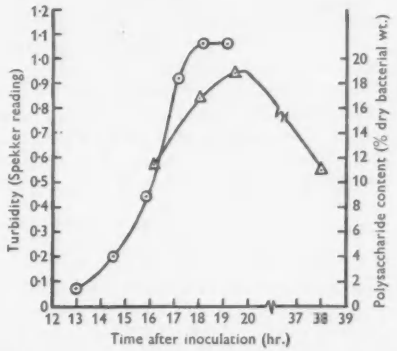

Fig. 2. Fluctuation of polysaccharide content of *Esch. coli* with time. Growth cycle followed turbidimetrically (○—○), and polysaccharide content (△—△) determined at various stages of growth and stationary phase.

Influence of factors determining onset of the stationary phase. Using the simple medium *A*, glucose concentration was maintained at 20 g./l. and the concentration of $(NH_4)_2SO_4$ varied. A linear relationship was obtained between stationary population and $(NH_4)_2SO_4$ concentration up to 1·2 g./l., the highest concentration used, at which the stationary population was approximately 10^9 bacteria/ml. Similarly, with excess $(NH_4)_2SO_4$ present at a concentration of 4 g./l., a linear relationship existed up to the highest concentration of glucose used (0·9 g./l.) and the stationary population was approximately $3{\cdot}7 \times 10^8$ bacteria/ml. These findings are in accordance with those of Dagley & Hinshelwood (1938) and Hinshelwood & Lodge (1939) for *Aerobacter aerogenes*, and indicate that over these ranges of foodstuff concentration the onset of stationary phase is determined by exhaustion of foodstuff and not by adverse conditions or toxic products. This was confirmed by following the consumption of glucose in the case of a medium for which glucose exhaustion during growth was anticipated and one for which glucose was in excess of growth requirements (Fig. 3).

In Fig. 3 the fall in turbidity in the stationary phase is clearly shown. From this information it was possible to follow the storage of polysaccharide during the growth of *Esch. coli* when, on one hand, glucose exhaustion determined onset of the stationary phase and, on the other hand, when the glucose was in excess of requirements. Results of these experiments are given in Table 2. It is evident that

the fluctuations of polysaccharide content in the stationary phase are not determined by glucose exhaustion.

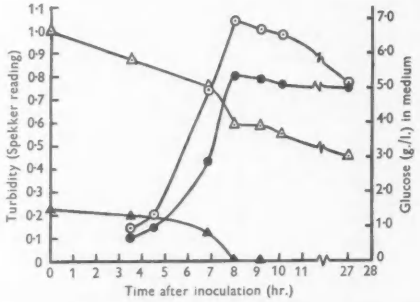

Fig. 3. Relation between growth of *Esch. coli* and amount of glucose in the simple ammonium salt-glucose medium *A*. (*a*) Excess glucose present: growth (○—○) glucose content of medium during growth (△—△). (*b*) Limiting concentration of glucose present: growth (●—●); glucose content of medium during growth (▲—▲).

Cessation of cell division and polysaccharide storage. When the stationary phase is reached the total bacterial mass, as measured by turbidity, does not remain constant, but invariably shows a steady decline. We compared the fall in polysaccharide content during the stationary phase with this fall in bacterial mass. It is seen, however, from Table 2 that storage of polysaccharide has not in every case reached its maximum when division ceases, and from Fig. 2 it is also seen that the maximal storage does not exactly coincide with the beginning of the stationary phase. Thus decrease in bacterial mass cannot be correlated with fall in polysaccharide content.

The effect of D*-phenylalanine on polysaccharide content of* Escherichia coli

Results using the method of acid hydrolysis. Dawson & Happold (1943), using the Jebbs strain of *Esch. coli*, reported that 0·01% (w/v) DL-phenylalanine (but not the L-isomer) caused an increase in cell polysaccharide grown in the amino-acid medium *B*. We sought to confirm this observation using both the simple and complex media and using the present method of polysaccharide determination. Of three flasks containing 1500 ml. simple medium *A*, 0·013% (w/v) D-phenylalanine was added to one, and growth curves were determined. To another, 0·013% (w/v) D-phenylalanine was added when the stationary phase was reached. Samples

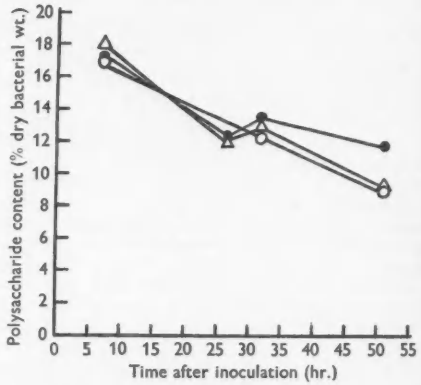

Fig. 4. Polysaccharide content of *Esch. coli* grown in presence and absence of D-phenylalanine on simple ammonium salt-glucose medium *A*; control, ○—○; 0·013% (w/v) D-phenylalanine added before inoculation, ●—●; and 0·013% (w/v) D-phenylalanine added when stationary phase reached (7·25 hr.), △—△.

Table 2. *Effect of limiting concentration of glucose and ammonium sulphate on stationary population and polysaccharide content of* Escherichia coli

Glucose (g./l.)	$(NH_4)_2SO_4$ (g./l.)	Stationary population (10^6/ml.)	Time after reaching stationary phase (hr.)	Polysaccharide content (% dry bacterial wt.)
(*a*) Onset of stationary phase determined by glucose exhaustion				
1·50	5·0	725	0	9·7
			19	13·4
			24	8·8
2·25	5·0	925	0	12·3
			19	12·2
			24	10·2
(*b*) Onset of stationary phase determined by $(NH_4)_2SO_4$ exhaustion				
5·0	1·2	737	0	14·0
			19	14·7
			24	11·1
5·0	1·8	950	0	11·6
			19	16·5
			24	11·5

(350 ml.) of cultures were withdrawn at intervals; the polysaccharide content of the cells determined, and these are shown in Fig. 4. D-Phenylalanine has no effect on growth rate or polysaccharide storage in this medium. The decline in polysaccharide content previously noted is shown in each case. The experiment was repeated using an inoculum of which about twenty-five generations of the cells had been grown in the medium containing 0·013% (w/v) D-phenylalanine. Here again there was no significant difference between cultures containing D-phenylalanine and those without.

Using the amino-acid medium *B*, to which glucose was added to a concentration of 1% (w/v), polysaccharide contents were estimated when turbidities showed in each case that the culture had just attained its stationary phase. Besides adding D-phenylalanine to two of the four flasks, 1% (w/v) tryptophan was also added to two of the flasks since this was present in the medium used by Dawson & Happold (1943). The results obtained again show no increase in polysaccharide content due to the presence of phenylalanine, e.g. control, 22·2%; 1% (w/v) DL-tryptophan added, 21·5%; 1% (w/v) DL-tryptophan + 0·02% (w/v) D-phenylalanine, 20·3% and 0·02% D-phenylalanine, 19·5% polysaccharide dry bacterial weight.

Polysaccharide content of various strains of Escherichia coli. Five different strains of *Esch. coli* were obtained through the courtesy of Dr D. E. Dolby of the Bacteriology Department of this University. They were grown in medium *B* containing 1% (w/v) glucose, and their polysaccharide contents determined in the usual way. It was found that there is a quite wide variation with strain, but none is as high as the strain used in this work. The polysaccharide contents were as follows: N.C.T.C. no. 5928, 21·2%; coli I, 10%; coli B, 6·9%; coli D, 9·5%; coli 4, 13·9% and coli 7148, 12·7%. The lowest storer was the 'B' strain which was a non-motile organism. It was decided, therefore, to see whether this organism (with a polysaccharide content similar to that of the Jebbs strain used by Dawson & Happold) showed an increased storage in the presence of D-phenylalanine, both in the presence and absence of glucose.

To four flasks containing 250 ml. medium B additions were made as follows: (1) 0·33% (w/v) glucose; (2) 0·33% (w/v) glucose + 0·013% (w/v) D-phenylalanine; (3) none; (4) 0·01% (w/v) D-phenylalanine. The polysaccharide content (% dry bacterial weight) of the 'B' strain grown on these four media was found to be (1) 8·2, (2) 8·1, (3) 3·3, (4) 3·3. Hence it was concluded that D-phenylalanine has no effect on the polysaccharide storage of *Esch. coli*. It is of interest to note that with this low-storing strain, as with the highest one, the addition of glucose to the medium causes a relatively large increase in stored polysaccharide. That low storage was not due to non-utilization of glucose was demonstrated by turbidity determinations during growth. The 'B' strain grew to approximately the same turbidity as the high-storing N.C.T.C. strain no. 5928.

Effect of various sugars on polysaccharide storage in the amino-acid medium

Effect of sugar concentration. Dawson & Happold (1943) reported that mannose had little effect on the polysaccharide storage of *Esch. coli* on the amino-acid medium *B* using the Jebbs strain of organism. We have repeated their experiments and, in addition, investigated the effect of the sugar concentration with both mannose and several other sugars.

To flasks containing 250 ml. amino-acid medium *B* (pH 7·5) increasing amounts of a sterile sugar solution were added. Each flask was inoculated with two loops of a culture, which had grown on the amino-acid medium containing the sugar in question, at a time when it had just attained its stationary phase. Growth was followed turbidimetrically and the cultures were centrifuged and washed when the stationary phase was reached. The effect of sugar concentration is given in Fig. 5. It will be seen that

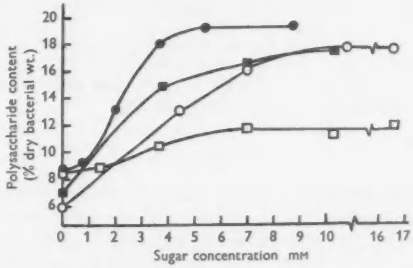

Fig. 5. Relation between polysaccharide content of *Esch. coli* and concentration of sugar present in amino-acid medium *B*; glucose, ○—○; lactose, ●—●; mannose, □—□; and galactose, ■—■.

a certain minimum storage of polysaccharide is obtained in the absence of sugar, but on the addition of it the stored polysaccharide increases to a maximum value, above which further additions of sugar produce no increase. It is also seen that, although some increase in storage was obtained when mannose and fructose (see Fig. 6) were present, the increase was markedly less than in the case of glucose, galactose and lactose.

Effect of pH. Hinshelwood & Lodge (1939), working with *Bact. lactis aerogenes*, have shown that stationary population is determined by the pH of the medium as well as by foodstuff concentration. At adverse pH values on the acid and alkaline sides of neutrality the total growth, and hence the foodstuff consumption, is greatly reduced. Accordingly, we have investigated the effect of pH on polysaccharide storage since this appears to depend on the amount of sugar metabolized. For each sugar at a concentration of 3·3 g./l. a range of media of pH between 6 and 8·5 was inoculated as before, the cells centrifuged when the stationary phase had just been reached and their polysaccharide content determined.

The dependence of polysaccharide content on pH for various sugars is shown in Fig. 6, where the

dependence of storage on pH is seen to be greatest in the case of the sugars for which the storage is highest.

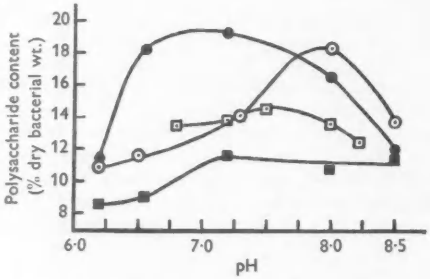

Fig. 6. Effect of pH on polysaccharide content of *Esch. coli* with various sugars at a concentration of 3·3 g./l. in amino-acid medium *B*; glucose, ○—○; galactose, ●—●; mannose, □—□; and fructose, ■—■.

DISCUSSION

It is sometimes assumed that the function of storage of polysaccharide by the cell is to serve as a store of carbohydrate for use when the other sugars in the medium are exhausted. Although we have found that there is a decline in storage during the stationary phase this is not directly related to the exhaustion of the normal supply of carbohydrate in the medium. Whilst it is possible that the polysaccharide may serve as a reserve source of energy, an alternative explanation for the fall in polysaccharide content may be found in the fact that increasing numbers of cells are dying in the stationary phase, and the decrease is reflected in autolytic activity. We cannot conclude from our results that the process of storage ceases exactly at the same time as cell division, whether the latter is due to exhaustion of glucose in the medium or not. On these grounds it is difficult to ascribe the fall in bacterial mass as reflected in the turbidity of the culture, which Monod (1942) first reported, and which we have repeatedly confirmed, entirely to dissimilation of stored carbohydrate.

With the present strain of *Esch. coli*, and using the method of estimation which we have developed, we have not observed any effect of small quantities of D-phenylalanine in increasing polysaccharide storage as reported by Dawson & Happold (1943). It would appear that the effect ascribed by Dawson & Happold to D-phenylalanine was, in fact, due to the method of estimation used. We conclude that the Sahyun (1931) method for glycogen determinations, coupled with reducing sugar determinations by the Hagedorn & Jensen (1923) technique, is unsuitable for bacterial polysaccharide determinations.

It is seen that although a low level of storage is obtained when the cells are provided with lactic acid and various amino-acids a greatly increased level is obtained when sugars are provided, particularly when pH is favourable to growth and storage. On the other hand, all sugars are not as effective as glucose or galactose; lower storage is obtained with mannose (which Dawson & Happold have previously commented upon) and fructose.

SUMMARY

1. The polysaccharide content of *Escherichia coli*, grown under various conditions, has been determined by acid hydrolysis of washed cell suspensions, followed by determination of reducing sugars by the method of Somogyi (1937).

2. The polysaccharide content falls during the stationary phase, but the onset of this fall does not exactly coincide with the cessation of cell division.

3. In a glucose-ammonium salt medium this trend is obtained whether exhaustion of nitrogen or exhaustion of glucose determines the onset of the stationary phase.

4. For these reasons the fall is not attributed to the utilization of stored polysaccharide by the cells when the sugar in the medium is used up.

5. For lactose, galactose and glucose, grown at pH 7·5 in an amino-acid medium which will support some growth in the absence of a sugar, the stored polysaccharide increases markedly with sugar concentration to a maximum, beyond which further additions cause no increase. With mannose and fructose little increase is produced.

6. At a fixed sugar concentration, variation of pH affected the storage of cells grown in glucose and galactose media. In these cases there are pH optima, which are not shown with the low-storing sugars, mannose and fructose.

7. There is a wide variation of stored polysaccharide content for different strains of *Esch. coli* grown under identical conditions.

8. The Sahyun (1931) method of glycogen determination, coupled with the Hagedorn & Jensen (1923) method for reducing sugars, is not satisfactory for the estimation of total bacterial polysaccharide.

9. The increased storage of polysaccharide, reported when this method of estimation was used and the cells were grown in the presence of D-phenylalanine, is not confirmed when the method described in paragraph 1 is employed.

We wish to thank Prof. F. C. Happold, at whose suggestion the research was undertaken and from whose advice we have benefited at various stages in its progress. We are indebted to Dr H. J. Rogers who drew our attention to the advantages of the method of estimation for bacterial polysaccharide which we adopted, and indicated possible sources of error in the Sahyun (1931) method. We have also benefited from discussions with Mr E. S. Holdsworth, who is undertaking similar investigations with *Corynebacterium diphtheriae*.

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Breakdown of the Oxidized Forms of Coenzymes I and II by an Enzyme from the Central Nervous System

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The enzymic breakdown of cozymase in various cells, and especially in tissues from the central nervous system, attracted our attention as being an unexpectedly rapid reaction (McIlwain & Hughes, 1948; McIlwain & Rodnight, 1949). It appeared that the breakdown, if unimpeded, would rapidly deplete tissues of this very important metabolite. Certain factors which would partly restrain the degradation under physiological conditions were observed, but the problem of the normal function of such a potent system remained unanswered. In general terms this appeared likely to be one of participation in, or control of, reactions in which cozymase functioned as coenzyme, and because of the importance of these the degrading enzyme has been studied further.

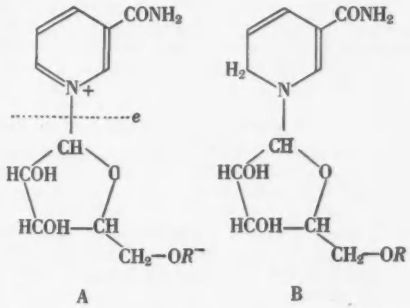

Fig. 1. A, coenzymes I or II; *e* indicates point of fission by brain preparations; B, or an isomeride with differently placed double bonds, dihydrocoenzymes I or II.

It is fairly well established that the breakdown of cozymase in nervous tissues and in certain micro-organisms takes place at the pyridinium-N (Fig. 1; Handler & Klein, 1942; McIlwain & Hughes, 1948). In some other tissues different processes preponderate. A link between nicotinamide and a substituted ribose is at present known to exist naturally only in cozymase (Co I), coenzyme II (Co II) and nicotinamide mononucleotide, although other natural and synthetic products approach these structures in various ways. The possible action of the enzyme on a number of such substances has now been examined.

EXPERIMENTAL

Materials

Cozymase. The specimens described previously (McIlwain & Rodnight, 1949) were used.

Dihydrocozymase. The preparation followed Schlenk (1941). Reaction with a specimen of Co I (120 mg., containing 68 mg. Co I) was complete in 30 min. and differential ethanol precipitation yielded a first fraction of 44 mg. dihydrocozymase. Titration with $K_3Fe(CN)_6$: 1·2 mg. required 0·45 ml. of 0·005 M-solution; content, 0·94 μmol./mg. Manometric reaction with $K_3Fe(CN)_6$: 4·3 mg. gave 4·65 μmol. CO_2; yield, 1·08 μmol./mg. Optical density at 340 mμ. (c = 0·094 mg./ml.), 0·501; content, 0·86 μmol./mg. The pure material as disodium salt contains 1·41 μmol./mg.

Two other specimens, of 45 and 55% purity, were also examined.

Coenzyme II was prepared from liver (in different batches, sheep and horse livers were used) following in part Altman's (1948) adaptation of the procedure of Warburg, Christian & Griese (1935). After precipitation as Hg and Ba salts, materials up to 10% pure were obtained. We are indebted to Dr B. C. J. G. Knight (Wellcome Physiological Research Laboratories) for a supply of horse liver immediately after slaughter.

Dihydrocoenzyme II. Attempted preparation from a coenzyme specimen of 10% purity, by the method used for

dihydrocozymase, was unsuccessful. Solutions of the substance were prepared enzymically, as described in the text below.

Nicotinamide glycosides. We are greatly indebted to Prof. A. R. Todd and Dr L. J. Haynes (University Chemical Laboratory, Cambridge) for specimens of these compounds in their dihydro forms. They were synthetic materials of which full details will be published elsewhere. Their oxidized forms were prepared by reaction with $K_3Fe(CN)_6$ in a manometric apparatus (see below).

Analytical methods

Ferricyanide and iodine titrations of dihydronicotinamide derivatives. With I_2, a 0·005 M-solution in KI was used and titrations performed in dilute acetic acid containing starch. With $K_3Fe(CN)_6$, a 0·005 M-solution was used, and the dihydro derivative was titrated in dilute acetic acid with KI and starch as external indicator.

Manometric reaction with ferricyanide (cf. Haas, 1937). The dihydronicotinamide derivatives were dissolved in 1 ml. of 0·02 M-$NaHCO_3$ saturated with 5% (v/v) CO_2 in N_2 in the main compartment of conical Warburg vessels of approx. 15 ml. The side arms of experimental and control vessels contained 0·3 ml. of 0·05 M-$K_3Fe(CN)_6$ which was tipped into the main compartment after equilibration with the above gas mixture; reaction was usually complete in 1 hr. This method was used also for reoxidizing dihydrocozymase to cozymase for use as substrate; the resulting solution, containing also the ferri- and ferro-cyanides, was employed as it had been established that such concentrations of the reagents had no effect on cozymase breakdown.

Nicotinamide and acid formation during reactions with the coenzymes were determined as described previously (McIlwain & Rodnight, 1949).

Spectrophotometric methods. The Beckman DU quartz spectrophotometer was used for determining dihydronicotinamide derivatives at concentrations of about 10^{-4} M and in 3 ml. of aqueous solution. Horecker & Kornberg's (1948) value of $6{\cdot}22 \times 10^6$ sq.cm./mol. was used for calculating concentrations of coenzymes I and II from the observed optical densities at 340 mμ. In demonstrating the stability of dihydrocozymase to the enzyme, reaction mixtures were examined between 320 and 400 mμ. before and after an incubation which inactivated 1·5 μmol. cozymase in a control experiment. No change in the absorption of 1·6 μmol. dihydrocozymase was found.

Determination of coenzyme II. This followed Adler, Euler, Günther & Plass (1939) and Ochoa & Weisz-Tabori's (1948) use of the *iso*citric dehydrogenase of mammalian heart. Sheep heart was treated according to Straub's (1942) procedure, and the acetone powder kept dry in a refrigerator. Portions of 0·5 g. of this were extracted weekly with 10 ml. of phosphate buffer (pH 7·3), the extract dialysed, kept cold, and used as *iso*citric dehydrogenase. DL-*iso*Citric acid was prepared according to Fittig & Miller (1898) and Nelson (1930).

In the determination, the following solutions were made to 2·95 ml. in spectrophotometer cells: 0·1 M-veronal buffer (pH 7·2) 1·0 ml.; 0·01 M-$MnCl_2$, 0·2 ml.; the Co II-containing solution and the dehydrogenase (0·05 ml.). The optical density at 340 mμ. was measured at room temperature (approx. 20°), readings being taken until values were steady, and 0·05 ml. M-*iso*citrate at pH 7·2 was then added. Readings were again taken, and from the change the volumes and the known extinction coefficient of dihydrocoenzyme II, its concentration was calculated. The course of an experiment of this type is illustrated in Fig. 2.

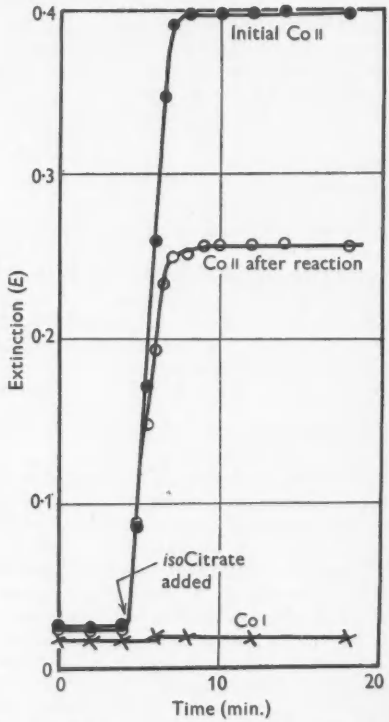

Fig. 2. Use of *iso*citrate system to show degradation of coenzyme II by a brain preparation. The initial and final Co II solutions (one-sixth of reaction mixture) are shown by the graph to contain respectively 0·86 and 0·56 μmol. of the coenzyme. The result with cozymase was from a vessel containing 1 μmol. cozymase, similarly diluted, and illustrates the specificity of the *iso*citric system and the purity of the cozymase.

Preparations of the cozymase-degrading enzyme

Tissue suspensions were made by grinding with sand, or by homogenizing, and were washed with saline. Aqueous extracts of washed tissue were made by repeated extraction of such suspensions with water. These have been described by McIlwain & Rodnight (1949). Preparations *SsDC* were made by extracting suspensions with strong salt solutions, dialysing and centrifuging. Data concerning them are given in the descriptions of the experiments in which they were used.

RESULTS

Dihydrocozymase

No change has been observed to be caused in dihydrocozymase by various brain preparations, and its much greater stability than cozymase in

nervous tissue is therefore noteworthy. The analytical methods used to detect possible change would in several cases have detected a reaction with a velocity less than 1% of that observed with cozymase. Table 1 shows that no reaction leading to liberation of nicotinamide was detectable, nor one leading to the formation (as with cozymase) or absorption of acid. Moreover, dihydrocozymase itself was determined in certain reaction mixtures, before and after incubation, by its absorption band at 340 mμ.; this showed less than 3% change. The dihydrocozymase was not pure, but three independently prepared specimens from three specimens of cozymase were examined. The initial cozymase specimens were degraded by brain preparations. Also, in one case the dihydrocozymase which had failed to react was reoxidized by ferricyanide and the resulting cozymase found to act as substrate. The dihydrocozymase specimen which failed to react did not inhibit the reaction with cozymase. Lack of reaction with dihydrocozymase thus cannot be attributed to effects of associated materials.

of whole brain tissue which had been ground with sand and thoroughly washed with 0·9% sodium chloride. It was, however, unstable in the presence of homogenized whole brain or blended heart muscle, aerobically, when oxidation to cozymase itself could be expected.

Nicotinamide glycosides

The most interesting of these compounds was the naturally occurring nicotinamide ribofuranoside. This contains the nicotinamide-ribose link which in cozymase is split during the present reaction. Nicotinamide riboside itself was, however, stable to the brain preparations used. Any reaction with three different enzyme preparations was of less than 1% of the speed with cozymase (Table 1). As criteria of reaction, appearance of nicotinamide (Koenig reaction) and acid formation were used.

In view of the stability of the riboside, it was not surprising that compounds less closely related to cozymase did not act as substrates. The mannoside, galactoside and glucoside were examined by the

Table 1. *Possible liberation of nicotinamide from its derivatives by brain preparations*

(All enzyme preparations were of ground tissues, washed with and diluted in 0·9% NaCl. Rates were determined from manometric observation of the course of evolution of CO_2 from bicarbonate containing solutions, and checked by nicotinamide estimation at the end of the reactions. The possible reaction with dihydrocozymase was also examined, with similar results, with two other preparations of grey and white matter from sheep brain, one from guinea-pig brain, and one from ox spinal cord. The riboside was examined also with preparations from white matter (sheep) and spinal cord (ox), and the mannoside, glucoside and galactoside with another preparation in addition to that quoted below; results were the same.)

Potential substrate ($M \times 10^{-4}$)	Source of enzyme preparation	Rate of reaction (μmol./mg./hr.)
Cozymase (5)	Whole sheep brain	0·95
Dihydrocozymase (5)	Whole sheep brain	<0·03
Cozymase (5)	White matter from centrum ovale of sheep brain (preparation A)	0·92
Dihydrocozymase (5)	White matter from centrum ovale of sheep brain (preparation A)	<0·008
Dihydrocozymase (5) with ferricyanide (11)	White matter from centrum ovale of sheep brain (preparation A)	0·84
Cozymase (3·8)	White matter from centrum ovale of sheep brain (preparation B)	0·96
Dihydrocozymase (4)	White matter from centrum ovale of sheep brain (preparation B)	<0·007
Dihydrocozymase (4) with cozymase (3·8)	White matter from centrum ovale of sheep brain (preparation B)	0·94
Cozymase (5)	Whole sheep brain	0·68
Nicotinamide ribofuranoside (16)	Whole sheep brain	<0·007
Nicotinamide mannoside (10)	Whole sheep brain	<0·007
Cozymase (5)	Sheep brain, white matter	1·13
Nicotinamide glucoside (10)	Sheep brain, white matter	0·01
Nicotinamide galactoside (10)	Sheep brain, white matter	<0·01

Dihydrocozymase added to brain preparations was found to be stable not only anaerobically, under which conditions most of the present reactions were carried out, but also aerobically when in the presence

methods of Table 1, and any reaction found to be less than 1% of that with cozymase. Dihydro derivatives of all four compounds were also examined, with a similar result.

Coenzyme II

This was found to be acted on by the enzyme only slightly less rapidly than cozymase. The reaction was first followed in bicarbonate-buffered mixtures at pH 7 by liberation of nicotinamide from a preparation containing about 4% of the coenzyme, and the rate of reaction found to be approx. 80% of that with cozymase. Purer coenzyme specimens, and various preparations of the enzyme, gave values between 70 and 85% of the rate with cozymase (Table 2; Fig. 3).

The rate of reaction was also followed by determination of the coenzyme II at different times during the reaction. Fig. 2 shows the determination of coenzyme II in an experiment in which this was done. Warburg vessels contained in their main compartments 0·9% NaCl and 0·01 M-$NaHCO_3$ (1 ml.) with a brain preparation *WS* (a sodium sulphate precipitate of a water extract of sheep brain washed with 0·9% NaCl, with activity of 25μ mol. cozymase degraded/hr./mg. N). The coenzyme II (1 μmol./0·2 ml., as material of 9% purity) was

Table 2. *Breakdown of coenzyme* II *by brain preparations*

(Reaction mixtures contained the coenzymes (1 μmol.), $NaHCO_3$ (22·5 μmol.), NaCl (155 μmol.) and brain preparations in 2·5 ml., with 5% (v/v) CO_2 in air at 37°. At intervals portions were removed, the reaction stopped with Zn salts, and nicotinamide determined as described by McIlwain & Rodnight (1949). The extract *SsDC* contained 1·2 mg. protein/ml. and 0·5 ml. was used in each experiment.)

Co II content of preparation (μmol./mg.)	Brain preparation	Rate of breakdown of coenzyme II (μmol./mg./hr.)	Rate of breakdown of coenzyme II (% of rate of cozymase in same experiment)
0·056	Guinea pig, mixed tissue ground and washed with NaCl	0·34	89
0·056	Ox spinal cord, as above	0·51 (μmol./vessel/hr.)	71
0·087	Sheep brain extract *SsDC*	1·40	77
0·087	Sheep brain extract *SsDC*	1·62	85

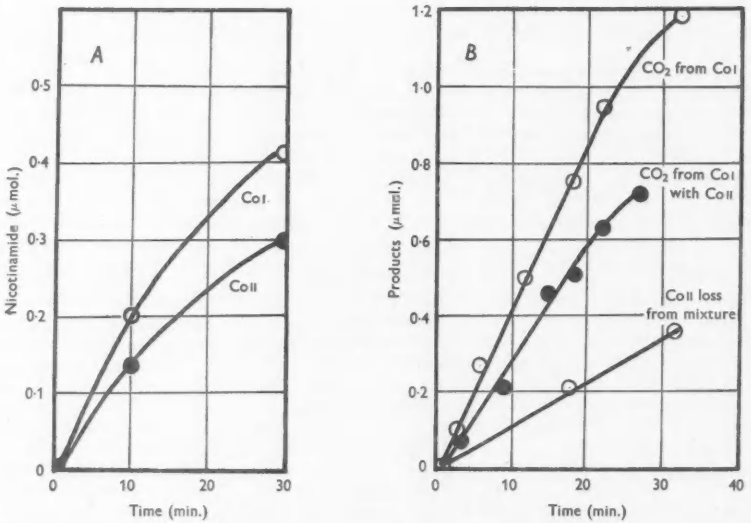

Fig. 3. Kinetics of coenzyme breakdown by brain preparations. A, nicotinamide liberated from coenzymes I and II, each 1 μmol. in 2·5 ml. NaCl-$NaHCO_3$-N_2/CO_2 (pH 7), by ground washed sheep spinal cord (equivalent to 8·3 mg. dry wt.) at 37°. B, CO_2 evolved during breakdown of cozymase (1·3 μmol.) in 1·4 ml. NaCl-$NaHCO_3$-N_2/CO_2 (pH 7) and of a mixture of cozymase and coenzyme II (total 1·3 μmol., in ratio 3/4) by a similar preparation of the degrading enzyme from ox spinal cord. Loss of coenzyme II from the same mixture is also shown.

contained in a side arm, yellow phosphorus in a centre well, and 5% (v/v) CO_2 in N_2 in the gas space. Other vessels contained cozymase, and others no substrate. After equilibration at 37°, reaction was started by tipping. Carbon dioxide evolution was followed and vessels removed at different times and put in ice water. To the portions taken for coenzyme II determination, $\frac{1}{3}$ vol. of 0·5 M-nicotinamide was added and specimens taken for test with the *iso*-citric system. From determinations which included that of Fig. 2, the rate of breakdown of coenzyme II was found to be 76% of that of cozymase.

The simultaneous degradation of the two coenzymes in a mixture containing them both in similar concentrations is shown in Fig. 3B. The results indicate that the two coenzymes compete for the degrading system.

Dihydrocoenzyme II

Demonstration of the stability of dihydrocoenzyme II in the presence of the enzyme was carried out as follows.

To 9·85 ml. of solution containing 1·16 μmol. Co II and the $MnCl_2$ and veronal buffer (0·1 M) of the *iso*citric system in a glass-stoppered tube, was added 0·15 ml. of the heart *iso*citric dehydrogenase. Sodium DL-*iso*citrate (0·1 M, 0·025 ml.) was added, and the optical density of a portion of the solution followed in a closed cell at 340 mμ. Another cell containing all reagents but no Co II was used as control. In 16 min. at room temperature the optical density became almost steady at a value corresponding to 1·06 μmol. of dihydrocompound; a further 0·005 ml. of the *iso*citrate was added, and the reading rose to correspond to 1·11 μmol. The whole solution was then heated quickly to 100° for 1 min., cooled in running water and centrifuged. The optical density of the solution compared with that of the control similarly treated fell by 10%, presumably due to the known instability of the dihydrocompound. Samples of this solution were used as source of dihydrocoenzyme II in the following experiment.

Spectrophotometer cells were prepared of which three contained the foregoing reaction mixture (2 ml.). To one of these was added 1 ml. of water; to two others a preparation *SsDC* of the cozymase-splitting enzyme from ox cord, as a clear solution. Another cell which served as control in the spectrophotometer contained the control solution of the previous paragraph (2 ml.) with the enzyme. Other cells contained the same control solution, enzyme and cozymase (0·27 μmol.). Change in the latter mixtures was determined by estimating nicotinamide; change in the former by spectrophotometric estimations at 340 mμ.

The results (Fig. 4) showed that the system rapidly inactivated cozymase (rate greater than 0·55 μmol./hr.), but caused very little change in dihydrocoenzyme II. The latter change in both control and experimental vessels was of about 0·02 μmol./hr.; any difference between these two due to an action of the enzyme itself on dihydrocoenzyme II was of less than 0·005 μmol./hr., i.e. less than 1% of the rate of reaction with cozymase. The small change observed in all vessels containing the dihydrocompound was presumably due to its instability in the reaction mixture in air at pH 7·2; no attempt had been made to remove dissolved oxygen initially present. In a vessel containing the enzyme in 3 ml. with both dihydrocoenzyme II (0·28 μmol.) and cozymase (0·18 μmol.), the dihydrocompound underwent no change other than that of the control, while cozymase was decomposed rapidly (Fig. 4). The experiment

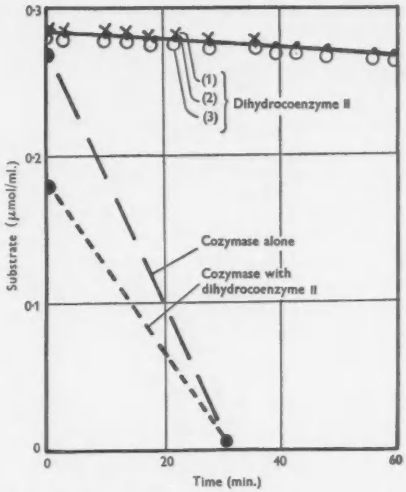

Fig. 4. Stability of dihydrocoenzyme II in the presence of the enzyme degrading cozymase. Above: dihydrocoenzyme II determined spectrophotometrically in three solutions (indicated by different points) which contained (1) no further additions, (2) and (3) the ox spinal cord preparation *SsDC* (1·5 mg. dry weight). Below: change in cozymase with the same quantity of cord preparation, with and without dihydrocoenzyme II.

was not adequate to give comparison of the rates of reaction with cozymase in the presence and absence of dihydrocoenzyme II, but these could not have differed greatly. Therefore, although nothing in the reaction mixture prevented the breakdown of cozymase, the dihydrocoenzyme II was stable.

Other N-alkyl heterocyclic compounds

Nicotinamide methosulphate and trigonelline (each 3×10^{-3} and 3×10^{-4} M) did not lead to detectable nicotinamide, nicotinic acid or free acid when incubated with sheep or guinea-pig brain preparations; reactions, 0·5% of the speed of that with cozymase, could have been detected. No acid was formed when the enzyme was incubated with benzimidazole methiodide or phenazine methosulphate at the same concentrations.

DISCUSSION

The major finding of the present experiments is that of the differential action of the cozymase-degrading enzyme on the oxidized, in distinction to the reduced, forms of the coenzymes. This is understandable when the structural differences between the two forms at the point of action of the enzyme are considered (Fig. 1). The enzyme acts on a bond at an ionized quaternary nitrogen atom. In dihydrocozymase this is replaced by a tertiary nitrogen atom presumably with an excess instead of a deficit of electrons. As the breakdown of cozymase is little if at all affected by the presence of either of the dihydrocompounds, these appear to have little affinity for the enzyme. The minimum structure compatible with activity of the enzyme has not been defined by the present experiments, but would appear to be at least a nicotinamide nucleotide (as distinct from nucleoside). The action of the enzyme at varying stages of purification on coenzyme II, as well as on cozymase, renders inappropriate the terms 'diphosphopyridine nucleotidase' and 'diphosphopyridine nucleosidase' which have been applied to it. These names, also, do not discriminate between the present enzyme and the cozymase pyrophosphatase of Kornberg (1948). Substrate competition in our experiments also indicates that the degrading enzyme from the central nervous system acts on both coenzymes.

The coenzyme molecules contain some fifty bonds of which eight or nine are of types which are readily split by various enzymes, for example, the bonds between ribose and phosphoric acid or between the carbon and nitrogen of the amide. It is therefore noteworthy that the present enzyme attacks the point at which the coenzymes differ most from their dihydro derivatives.

The effects of this differential attack seem likely to be far reaching (McIlwain, 1949*a*, *b*). They can immediately be expected to include changes in redox potential and in systems sensitive to such changes, and modifications of equilibria involving coenzyme-catalysed dehydrogenases. Inhibition of cozymase breakdown by low concentrations of substances (including phenosafranine) which disturb the Pasteur effect (McIlwain, 1949*b*) suggests that the system degrading cozymase does indeed play a central part in carbohydrate metabolism in normal tissues, possibly participating in the phosphorylation associated with tissue oxidations (cf. Judah & Williams-Ashman, 1949). It is noteworthy that in brain the speed of cozymase degradation was found (McIlwain & Rodnight, 1949) to be 2·2–3·3 times that of the oxygen uptake, both changes being expressed in molar quantities. The ratio appears to be similar in muscle and the enzyme exists in many animal tissues (Spaulding & Graham, 1947) although at a lower level.

SUMMARY

1. Preparations from the mammalian central nervous system, which rapidly inactivated cozymase, inactivated also coenzyme II at 70–85% of the rate of their reaction with cozymase.

2. Inactivation of coenzyme II, like that of cozymase, was accompanied by liberation of nicotinamide.

3. Competition took place between the two coenzymes for the degrading system, and presumably, therefore, a common enzyme was responsible for the two reactions.

4. Nicotinamide ribofuranoside, nicotinamide glycosides less closely related to cozymase, and several other nicotinamide derivatives were not affected by the enzyme.

5. Dihydrocoenzymes I and II were not affected by the enzyme, and did not affect the breakdown by it of coenzymes I and II.

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Apparent Vitamin C in the Walnut (*Juglans regia*)

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A previous communication (Wokes & Melville, 1948) described studies carried out over several years on the seasonal distribution of vitamin C in different tissues of the fruit and other parts of the walnut tree. The results showed that the translocation of vitamin C as such from the epicarp, leaf mesophyll and other photosynthetic centres, where the vitamin is probably synthesized, to the endocarp, where it occurs in such remarkably high concentration, seems to involve its passage up a steep concentration gradient presenting unusual difficulties as compared with the leaf to fruit gradients in most other species which have been investigated. In our studies on the walnut, apparent vitamin C was necessarily determined in all the samples, since the results for true vitamin C were determined generally by subtracting the figure for apparent vitamin C from that for total vitamin C. However, the data for apparent vitamin C were omitted from our previous paper in order to demonstrate the type of picture which is obtained if the presence and possible functions of the apparent vitamin C are ignored. The present paper supplies the missing data, enlarging and clarifying the picture.

Gerghelezhiu (1937), who discovered the high concentration of vitamin C in unripe walnut fruits, did not seem to realize that his raw materials could contain considerable amounts of dye reductants which were not ascorbic acid, but this may have been due to his use of sulphur dioxide in the extracting fluid which, as will be shown later, can prevent the apparent vitamin C from reacting with the dye. His findings were confirmed by Pyke, Melville & Sarson (1942) and by Ranganathan (1942), but neither paper reported the presence of an appreciable amount of apparent vitamin C in the unripe walnuts examined, perhaps because these were too mature. Ranganathan (1942) stated that tests for interfering substances showed these to form 4–6% of the total vitamin C. Taylor (1943) reported the presence of about 13% of interfering substances in the total vitamin C in some unripe walnuts which he had examined. Melville, Wokes & Organ (1943) found the total vitamin C in six unripe fruits of *Juglans regia* and fifteen of allied species, to contain 12–73% of interfering substances, for which they suggested the provisional term 'apparent vitamin C'. They used a modification of Lugg's (1942) formaldehyde method (Wokes, Organ & Jacoby, 1943) in which the residual apparent vitamin C, after destruction of the true vitamin C by formaldehyde at pH 4·5, was titrated with the dye at a pH below 1.

Mapson (1943) found that some 14% of the total dye titration for walnuts preserved in syrup was due to non-specific reductants to which he applied the term 'reductones'. In his modification of Lugg's (1942) method, the 'reductones' were titrated at pH 0·6 and total vitamin C at pH about 1·2 (except when the presence of sulphides, sulphites or thiol compounds was suspected). His assumption that the apparent vitamin C in walnuts consisted of reductones was understandable in view of the finding of Wokes, Organ, Duncan & Jacoby (1943) that the apparent vitamin C in molasses, malt extract and certain fruit juices after prolonged storage closely resembled reductone or reductic acid in chemical and physical properties. Nevertheless, the apparent vitamin C in walnuts cannot be reductone, since its dye titration value is very low at pH 2 and increases markedly as the titration pH is lowered (Wokes, 1946*a*), in contrast with reductone, the dye titration value of which decreases as the titration pH is lowered (Martius & von Euler, 1934; Wokes, 1946*a*). The use of too high a titration pH probably explains the failure of Lugg & Weller (1943) and of Klose, Peat & Fevold (1948) to discover much apparent vitamin C in unripe walnuts, though it should be added that the latter workers complicated their results by using sulphur dioxide in the extracting fluid. On the other hand, when Tuba, Hunter & Osborne (1947) applied to walnuts Levy's (1943) method of estimating ascorbic acid in the presence of reductones and other interfering substances they found 24–38% of the total vitamin C to be non-specific dye reductants.

METHODS

Collection and dissection of material was as described previously (Wokes & Melville, 1948).

Estimation of apparent vitamin C

(*a*) *Formaldehyde method.* The visual method described by Wokes, Organ & Jacoby (1943) was used, taking care to add after the formaldehyde treatment sufficient 25% (w/v) HPO_3 to ensure that the pH was brought down to 0·5–0·7

before titrating. (This is the pH of the reaction mixture when determining total vitamin C.) The pH of the titration mixture was determined and the result corrected to pH 0·7 by means of the curve previously published (Wokes, 1946*a*). When calculating from the dye readings the concentration of apparent vitamin C, its dye factor was assumed to be the same as that of ascorbic acid. This procedure, the most convenient when estimating true vitamin C by difference, leaves the results for apparent vitamin C open to correction when its chemical nature is established. It does not, however, affect their relative values. When sufficient material was available, estimations of total and apparent vitamin C were occasionally made by the potentiometric method (Wokes, Organ & Jacoby, 1943). This always gave results in good agreement with the visual results, and also provided information on the effect of the titration time on the dye titration value (see p. 350). The fact, repeatedly confirmed, that the dye titration value increased as the pH was reduced below 1·5 gave strong reason for believing that the apparent vitamin C was not reductone.

(*b*) *By using ascorbic acid oxidase.* This enzyme, derived from cucumber juice, was found to destroy the whole of the ascorbic acid in walnut extracts without exerting any appreciable effect on the apparent vitamin C. To ensure that the destruction of the ascorbic acid was complete, the mixture was incubated with the enzyme at pH 5 and at 38–40° until dye readings on samples became constant. Recovery experiments carried out with different levels of added ascorbic acid gave similar results. The data obtained in a typical experiment are plotted in Fig. 1. From the general shape of the curves, it was clear that the enzyme had ceased to act on any dye reductant after about 20 min., either in the recovery or in the test experiment. The amount of apparent vitamin C then left in the two reaction mixtures was practically the same, indicating 349 and 344 mg./100 g. respectively in the original leaves, as compared with about 320 mg./100 g. by the formaldehyde method. Hence it was assumed that the enzyme had exerted no appreciable effect on the apparent vitamin C. Since ascorbic acid oxidase acts slowly on reductone, further evidence was thus provided that the apparent vitamin C was not reductone. The method was not applied to many of the samples because it was tedious and required large amounts of material.

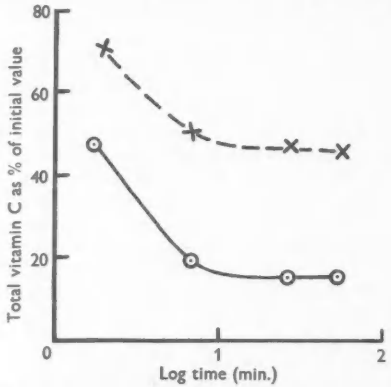

Fig. 1. Use of ascorbic acid oxidase to differentiate apparent from true vitamin C in walnut leaves. Results obtained on extract of leaves, ×– –×; results obtained on extract plus added ascorbic acid, ⊙——⊙.

(*c*) *By differential titration.* Since the dye titration value of the apparent vitamin C in walnuts falls to zero as the titration pH is raised above 2, it is possible to titrate the true vitamin C as such at a pH of about 3, then to add excess of HPO_3 to bring down the pH to 0·6 or 0·7 and continue the titration to determine the apparent vitamin C. In practice this method was found less convenient and accurate than the formaldehyde method, and was applied only on a few occasions, when it gave fair agreement with other methods.

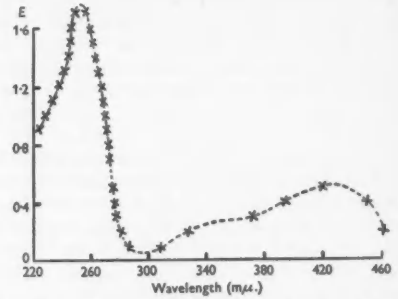

Fig. 2. Absorption curve of the yellow colour obtained when titrating walnut extracts with indophenol dye.

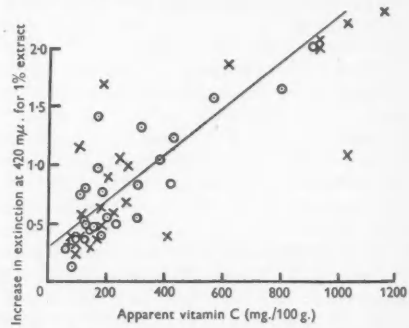

Fig. 3. Correlation of concentration of apparent vitamin C in walnut tissues with increase in yellow colour (measured spectrophotometrically at 420 mμ.) when extracts of the tissues were titrated with indophenol dye. Results on fruit tissues indicated thus ⊙; results on stem and leaf indicated thus ×. Regression line calculated from the equation $y = 0{\cdot}9618 + 0{\cdot}002047\,(x - 333)$.

(*d*) *By increase in yellow colour.* During titration of walnut extracts containing apparent vitamin C a yellow colour develops. Spectroscopic examination of this yellow colour revealed a broad absorption band in the visible spectrum with a maximum at about 420 mμ., and a narrow band of four or five times the intensity with a maximum at about 250 mμ. (see Fig. 2). Using the Hilger-Nutting spectrophotometer, we determined the increase in yellow colour at

420 mμ., caused by the dye titration, employing the cross-over technique (Wokes & Still, 1942). The results, expressed as increase in $E^{1\%}_{1\,cm.}$, plotted against the concentration of apparent vitamin C (in mg./100 g.), determined by the formaldehyde method, are shown in Fig. 3, together with the regression line. The correlation coefficient for these data, $r=0{\cdot}83$, was highly significant ($t=14{\cdot}14$, $P<0{\cdot}01$) and is strong evidence that apparent vitamin C usually consists of a single compound which is converted into a yellow pigment on oxidation. Nevertheless, occasional aberrant results preclude the use of this yellow colour for a routine estimation.

Loss of apparent vitamin C during dissection. In a number of experiments, the weights of apparent vitamin C in the dissected parts of leaflets and nuts were compared with the average yield from whole leaflets and nuts from the same spray. Losses due to dissection were found to be no greater than for true vitamin C in the same material (cf. Wokes & Melville, 1948). For example, in samples of leaves collected on 10 different days between May and September the weighted mean concentration of apparent vitamin C in dissected leaflets ranged from 85 to 136 and averaged 107% of the concentration found in whole leaflets ground without dissection; but in samples collected on 3 different days in October the corresponding percentages were 82, 71 and 44. Nuts were more variable, owing to a greater sampling error. For example, two dissected nuts gave 177 and 267 mg./100 g., averaging 222 mg./100 g., which was in reasonable agreement with the mean of 214±23 mg./100 g. obtained for ten whole nuts from the same sample, but the results on the individual nuts ranged from 131 to 382 mg./100 g. It is probable that oxidizing enzymes capable of destroying apparent vitamin C are present in walnut tissues, since, on grinding with water and quartz sand, a yellow colour developed similar to that formed during titration. At the same time there was a rapid drop in the dye titration value. On the other hand, the apparent vitamin C in HPO_3 extracts of these tissues was fairly stable during storage in brown bottles under N_2 in the refrigerator, losses as low as 1–2%/month being frequently found after more than a year's storage, although considerably greater losses could occur if there was much exposure to air.

RESULTS

Whole fruits (nuts)

A seasonal trend in the concentration of apparent vitamin C in entire fruits is fully established, although the results are more variable than those previously reported for true vitamin C. Data on nearly 100 fruits from samples collected June–August 1944, May–August 1945 and June 1946, are summarized in Table 1. The highest concentration of apparent vitamin C was found in the youngest fruits weighing not more than 1 g., and was usually between 800 and 1000 mg./100 g. As the fruits grew, the concentration fell until at maturity it was only 0·2–0·1 of the initial value. The concentration of apparent vitamin C was therefore already at its height when the earliest samples were taken, whereas

Table 1. *Seasonal changes in concentration of apparent vitamin C in walnut fruits*

Sample no.	Date gathered	Average wt. of fruits (g.)	Apparent vitamin C (mg./100 g.)		Apparent vitamin C	
			Individual fruits	Mean	(mg./fruit, mean)	(As % of total vitamin C)
			From main tree			
1	7 June 1944	1	Pooled	800	8	62
2	13	1	Pooled	847	9	47
3	21	3	760, 715, 658	711	21	26
4	26	3·6	540, 520, 440, 430	483	17	19
5	4 July 1944	10	405, 325, 315	345	35	11
6	12	20·3	220, 275, 225, 320	260	51	14
7	18	17·5	185, 215, 120, 210	180	32	11
8	25	22·6	235, 195, 160, 248	210	48	13
9	1 Aug. 1944	24·9	155, 150, 120, 115	135	36*	11
10	9	32·1	135, 148	141	45*	14
11	22	21·7	190, 140	165	36	12
25	17 May 1945	0·3	Pooled	789	2·4	46
26	30	0·5	716, 475, 539	577	3	35
27	6 June 1945	1·75	568, 501	535	9	23
28	12	2·8	401, 323, 380	368	10	16
29	19	4·1	429, 308, 384	348	14	17
30	26	14·0	243, 205, 234	227	32	14
31	10 July 1945	25·2	197, 168, 139	168	42	15
32	23	29·7	158, 174, 172	168	50	14
33	8 Aug. 1945	32·0	87, 100, 103	97	31	9
45	18 June 1946	0·3	Pooled	525	1·5	43
			From other trees			
2*k*2	29 June 1944	10·6	455, 405, 390	417	44	17
2*k*3	25 June 1945	18·5	223, 140, 200 214, 194, 167	190	35	10

* Excluding woody portion.

the peak value was not reached for true vitamin C until about a fortnight later and then fell gradually as lignification of the endocarp proceeded. The occurrence of peak values for these substances at different times at first suggested that apparent vitamin C might be a precursor of ascorbic acid in the plant. This idea had to be abandoned later when evidence was found that apparent vitamin C is related to juglone. The total amount of apparent vitamin C in a single fruit increases during growth from about 2 mg. to 50 mg., whereas true vitamin C increases much more rapidly from about 3 mg. to over 300 mg. per fruit. Thus, there is finally only about one-sixth as much apparent as true vitamin C. The change in proportion does not suggest any close physiological link between these substances in the plant, and no correlation was found between the observations (1944) of the true and apparent vitamin C in entire fruits. There was, however, a high correlation ($r = 0{\cdot}99$) between apparent vitamin C and true vitamin C a fortnight later ($t = 28{\cdot}37$, $P < 0{\cdot}01$). This result may be due to parallel trends in unrelated processes rather than to a causal relation.

Separate tissues of the fruits

Fruits were dissected as previously described and the separated epicarp, mesocarp, endocarp and kernel were assayed for both true and apparent vitamin C, the same fruits providing data for the whole fruit and separate tissues.

Initial high concentrations (well over 1 g./100 g.) of apparent vitamin C were found in the epicarp and mesocarp (Table 2). These values declined as growth proceeded, but there was a final small rise in the full-grown fruits. The concentrations in the endocarp were much lower, but in general showed similar trends. A definite concentration gradient was generally demonstrable from the epicarp and mesocarp towards the endocarp, and there was always a clear gradient into the testa and its contained endosperm or embryo. Often no positive gradient could be shown from the entire fruit stalk into the endocarp, but, in contrast with true vitamin C, a gradient through the phloem could usually be established as the concentration of apparent vitamin C was higher than in the other tissues. The final rise in concentration in the endocarp was greater if the non-lignified portion alone was considered. This could be explained by a differential partition of the apparent vitamin C at a stage when there was little difference in concentration between epicarp, mesocarp and endocarp. Correlations between true and apparent vitamin C in the fruit tissues were investigated, but the only significant correlation found was negative, between true and apparent in the epicarp ($r = -0{\cdot}93, t = 5{\cdot}02, P < 0{\cdot}01$). At first this was interpreted as evidence that apparent vitamin C was a precursor of ascorbic acid, but later work, indicating a relationship to juglone, appears to rule out that view. The same difficulty arises with

Table 2. *Seasonal distribution of apparent vitamin C in different tissues of walnut fruits*

(Dates of collection of samples are given in Tables 4 and 7.)

Sample no.	Fruit stalk	Apparent vitamin C (mg./100 g.) Epicarp	Mesocarp	Endocarp	Testa	Endosperm
1	229	1616	—	92	—	—
2	—	1497	—	203	—	—
3	—	712	924	102	—	—
4	120	595	1368	185	—	—
5	145	405	610	65	20	—
6	—	195	265	150	5	1
7	—	125	275	125	5	1
8	—	150	240	110	5	1
9	200	120	200	100*	9	1
10	120	130	145	160*	27	1
11	125	240	320	210*	12	1
26	106	1029	623	112	—	—
27	95	716	1094	117	—	—
28	106	383	802	111	—	—
29	81	428	919	134	—	—
30	63	145	411	82	—	—
31	134	102	235	139	—	—
32	108	185	122	129	3†	
33	88	69	112	204	—	
34	30	167	229	—	—	
2*1*	—	450	780	78	—	
2*2*	—	265	715	30	10†	
2*3*	—	215	361	66	8†	

* Excluding woody portion of endocarp.
† Result on whole kernel.

the correlation ($r = 0{\cdot}87$) found between apparent vitamin C in the epicarp and true in the endocarp a fortnight later ($t = 4{\cdot}85$, $P < 0{\cdot}01$).

Leaves

Data on leaves are given in Table 3. Results on twenty-one samples show that the concentration of apparent vitamin C was always highest in the mesophyll, where there was no definite seasonal change, whereas the concentration of true vitamin C fell gradually with age. While the fruits were growing, there was a clear concentration gradient of apparent vitamin C from mesophyll through the leaf vascular tissue and rachis as far as the leaf base. Hence a gradient into the main stem was more difficult to establish, especially from older leaves, despite attempts to allow for the proportion of phloem in the tissue examined.

Table 3. *Apparent vitamin C in walnut leaves*

(Dates of collection of samples are given in Tables 4 and 7.)

Sample no.	Apparent vitamin C (mg./100 g.)					
	Whole leaflets	Mesophyll	Vascular tissue	Rachis	Petiole	Main stem, cortex and phloem
9	250	—	—	—	120	—
11	—	340	160	—	120	—
25	243	299	233	260	119	91
26	242	231	150	141	94	132
27	215	206	182	116	87	173
28	243	277	171	95	95	151
29	271	411	182	109	106	259
30	144	169	95	91	48	304
31	204	349	116	83	81	245
32	192	226	94	94	72	459
33	224	325	118	106	87	536
34	427	410	310	158	51	546
35	243	—	—	37	36	97
36	360	298	291	52	76	179
37	234	68	116	116	68	156
38	202	235	117	64	36	500
43	306	—	—	—	—	412
44	270	542	363	235	—	232
45	195	203	199	162	136	123
46	192	211	170	111	131	169

Buds, catkins and main stem

Data obtained from August 1944 to October 1946 are given in Table 4. The concentration of apparent vitamin C in the buds often exceeded 1 g./100 g., but varied widely from time to time. This was not entirely due to the sampling error, since the results on vegetative buds were usually similar to those on reproductive buds in the same sample. In the early summer, when the reproductive buds began to develop into female catkins, there was a considerable increase in their size so that, although the total amount of apparent vitamin C in an average bud had increased, its concentration remained steady or even fell. On the other hand, the concentration of apparent vitamin C in the vegetative buds usually increased in the early summer, shortly before they opened, but fell again rapidly as the tiny leaves grew. When the male catkin buds were just distinguishable in late summer, the concentration of 2–3 g./100 g. was higher than in any other plant tissue, but fell slowly during the autumn. Simultaneously, their average weight increased from about 50 to 80 mg., so that the amount of apparent vitamin C in a catkin bud remained at about 1 mg. In the spring, the male catkins grew rapidly till they weighed more than 1 g. each. The concentration of apparent vitamin C fell steadily to less than 500 mg./100 g. The amount in an average catkin fell slightly at the beginning of the spring growth, but then increased to 3 or 4 mg. As the catkins now contained a good deal of fresh green tissue, it is possible that some of this apparent vitamin C was formed by photosynthetic activity in the catkins, and was not translocated from other parts of the tree. Since the concentration in the adjacent stem, cortex and phloem at this time was 300–500 mg./100 g., and about 5% of this consisted of sieve tissue, a comparatively small differential concentration in the phloem would have ensured a positive translocation gradient into the catkins. At other times of the year, the observed concentrations were less favourable to direct translocation into any type of bud, though it is not impossible that a positive gradient always existed in the phloem serving these buds. The data on the cortex and phloem from different parts of the stem showed that from October to April the concentration gradient of apparent vitamin C was con-

Table 4. *Seasonal distribution of apparent vitamin C in buds, catkins and stem*

(C.Y.G. = Current year's growth. P.Y.G. = Previous year's growth.)

Sample no.	Date gathered	Apparent vitamin C (mg./100 g.) Stem (cortex and phloem) C.Y.G.	P.Y.G.	Older	Buds Vegetative	Reproductive	Male catkins	Apparent vitamin C (mg./catkin)
12	29 Aug. 1944	215	—	—	—	995	2530	1·3
13	4 Sept. 1944	—	—	—	988	1040	2430	1·3
14	11	—	—	—	970	813	—	—
15	10 Oct. 1944	157	—	—	728	785	1485	1·1
16	14 Nov. 1944	795	—	—	1200	1040	2690	1·6
17	20	580	—	—	1220	—	2310	1·5
18	27	335	—	—	1080	1180	2130	1·3
19	8 Jan. 1945	340	—	418, 369	711	—	1630	0·8
20	21	297	195	119, 156	472	604	1467	0·9
21	28 Feb. 1945	310	264	174, 61	435	273	1146	0·9
22	13 Mar. 1945	444	202	201	627	575	762	0·9
23	4 Apr. 1945	306	—	—	371	568	481	3·4
24	13	—	—	—	—	—	488	4·3
25	17 May 1945	91	369	—	1022	776*	—	—
26	30	132	143	—	1190	577*	—	—
27	6 June 1945	173	409	523, 502	1132	—	—	—
28	12	151	360	—	935	615	—	—
29	19	259	287	434	1033	—	—	—
30	26	304	424	—	1033	—	—	—
31	10 July 1945	245	309	—	936	—	—	—
32	23	459	481	—	951	—	—	—
33	8 Aug. 1945	536	576	—	1051	—	—	—
34	11 Sept. 1945	546	446	346, 382	1214	—	2592	—
35	2 Oct. 1945	97	187	130, 87, 50, 23	964	1045	1919	—
36	9	179	180	183	292	—	1041	0·7
37	15	156	149	—	454	—	1303	0·9
38	22	500	156	—	695	—	1878	1·4
39	31	323	176	—	616	—	1674	1·2
40	13 Nov. 1945	269	201	—	817	—	1617	1·1
41	15 Jan. 1946	291	212	—	769	—	1535	0·9
B	21 Mar. 1946	252	—	—	—	—	—	—
42	2 Apr. 1946	189	119	—	499	500	699	1·4
43	15	412	223	—	412	—	354	2·9
44	29	235	259	—	581	—	198	—
45	18 June 1946	123	388	—	—	—	—	—
46	1 July 1946	169	303	—	1043	—	—	—
47	23 Sept. 1946	—	—	—	—	—	2200	0·6
48	24 Oct. 1946	100	179	248	365	—	1200	0·9

* Excluding woody portion.

sistently from new to older tissue and could be traced some considerable distance down the stem. From May to September the observed concentration gradient was consistently in the reverse direction, from older to newer tissue. In March 1945 we estimated the apparent vitamin C in all parts of a young walnut sapling. Our results (see Table 5) showed a concentration gradient from the cortex plus phloem of the previous year's growth down into the fine roots, where, however, there was still about 300 mg./100 g., several times the concentration of true vitamin C. The concentration in the cortex plus phloem of the current year's growth was lower than in the previous year's growth, but this may have been due to a higher proportion of cortex in the former. The value for the previous year's growth was checked by a control experiment with added ascorbic acid on a second sample. The result (386 mg./100 g.) was in fair agreement with the previous result of 415 mg./100 g. and showed that the whole of the true vitamin C was being destroyed by the formaldehyde under the experimental conditions.

Table 5. *Apparent vitamin C in walnut sapling*

	Apparent vitamin C (mg./100 g.)	(As % of total)
Buds	596	70
Stem (cortex plus phloem):		
Current year's growth	298	54
Previous year's growth	415	69
Roots	327	71
Fine roots	302	76

Storage experiments

From the evidence of concentration gradients apparent vitamin C seemed much more likely than true vitamin C to be translocated from leaf to fruit during the growth of the latter. This led to the hypothesis that the apparent vitamin C, after its arrival in the endocarp, may yield true vitamin C for which it has served either as a precursor or as a carrier. In order to test this hypothesis, we carried out in 1944 a storage experiment with some immature walnuts which, after sampling for assay, were stored in the refrigerator at 4–5° in a box with access to air. The results (Table 6) showed, after 16 days' storage, an increase of 302 mg./100 g. in the concentration of true vitamin C and a decrease of 36 mg./100 g. in the concentration of apparent vitamin C. (Allowance is made in these figures for the gradual loss in weight of the fruits during storage.) Unfortunately, owing to the large sampling error, these differences did not reach significance. Moreover, the loss of apparent vitamin C was very much smaller than the gain of true vitamin C. Results on dissected tissues were also inconclusive, the data indicating a greater loss of true than of apparent vitamin C throughout. Ascorbic acid oxidase liberated during dissection may have caused this loss as the tissues had shrunk and become more brittle during storage. This view was supported by the observation that the mean concentration of true vitamin C in the fruits dissected was about 20% lower than that in whole fruits. In 1945, a further storage experiment was made, extending over a period of 2 months (Table 7). The results, corrected for changes in weight during storage, showed no significant changes in the concentrations of either true or apparent vitamin C during the period, thus corroborating the experiment of 1944.

Table 6. *Changes in average concentration of true and of apparent vitamin C in walnut fruits during storage*

(Storage for 16 days at 4–5° in July 1944; all results given as mg./100 g.)

	Whole fruit		Epicarp		Mesocarp		Endocarp		Kernel	
	True	Apparent	True	Apparent	True	Apparent	True	Apparent	True	Apparent
After storage	2337	379	1025	233	963	704	3046	58	35	2
Before storage	2035	417	1195	265	1085	715	3140	30	140	10
Increase or decrease during storage	+302	−38	−170	−32	−122	−11	−94	+28	−105	−8
S.D. of mean (after storage)	171	37	17	29	31	61	4	10	—	—
S.D. of mean (before storage)	11	20	—	—	—	—	—	—	—	—

Table 7. *Average concentrations of true and of apparent vitamin C in whole walnut fruits at intervals during storage*

(Storage 4–5° in 1945; all results given as mean ±S.D.)

Date examined	Period of storage (days)	No. of fruits examined	Vitamin C (mg./100 g.) True	Apparent
29 June	0	6	1849± 57	193±15
5 July	6	4	1910±138	243±51
13	14	4	1363±159	196±28
19	20	4	1869± 37	238±15
7 Aug.	39	4	1798± 90	198±33
27	59	4	1815± 38	180±30

Extraction experiments

Apparent vitamin C was found to be readily soluble in ethanol and aqueous solvents, but much less soluble in solvents immiscible with water, such as benzene, light petroleum, methyl or ethyl ether, chloroform, *iso*butanol, carbon tetrachloride, ethyl acetate and carbon disulphide. Thus, purification by phase separation seemed unpromising. Whilst it could be extracted from fresh walnut tissues by grinding with ethanol and quartz sand, this often led to heavy losses which could be avoided by adding a small amount of hydrochloric acid to the solvent (2–5% of concentrated acid by volume). The extracts contained much chlorophyll, which rendered visual dye titrations difficult. The chlorophyll could be removed by shaking with light petroleum, leaving a pinkish yellow solution of apparent vitamin C in the acid ethanol. The acid could be removed by evaporation *in vacuo*, yielding very unstable residues with initial concentrations of apparent vitamin C as high as 7%.

Rate of reaction with indophenol dye

One of the most characteristic properties of apparent vitamin C is its slower rate of reaction with the dye, compared with the almost instantaneous reaction of the latter with ascorbic acid. The slower rate can be detected in visual titrations, but is particularly well marked in potentiometric estimations. Wokes, Organ & Jacoby (1943), using a modification of the potentiometric method of Harris, Mapson & Wang (1942), showed that the amount of apparent vitamin C found in a stored sample of dehydrated carrot could vary from 20 to 90 mg./100 g. as the time taken to carry out a titration was

increased from about 2 to 30 min. This observation justifies the importance we have attached to the control of the titration time. Attempts have been made to estimate true vitamin C in the presence of these interfering substances by adding a known excess of dye and measuring photoelectrically at short intervals (e.g. 15, 30, 60 sec. after the dye addition) the amount of unreduced dye. The readings are plotted against time and extrapolated back to zero time to estimate the true vitamin C. The interfering substance could then, if desired, be estimated by difference, assuming that the reaction spectively about half and a fiftieth of that for ascorbic acid, and utilized these in a method of estimating ascorbic acid in the presence of reductones which gave good agreement with the formaldehyde method. Their method would not, however, distinguish between ascorbic acid and *iso*ascorbic acid or hydroxytetronic acid, because the velocity constants of these acids with the dye at pH 1·4 and at 3·5 were too close to one another. Such differentiation could be effected by the infrared absorption method of Trotter, Thompson & Wokes (1948), but this method was not available for the work described

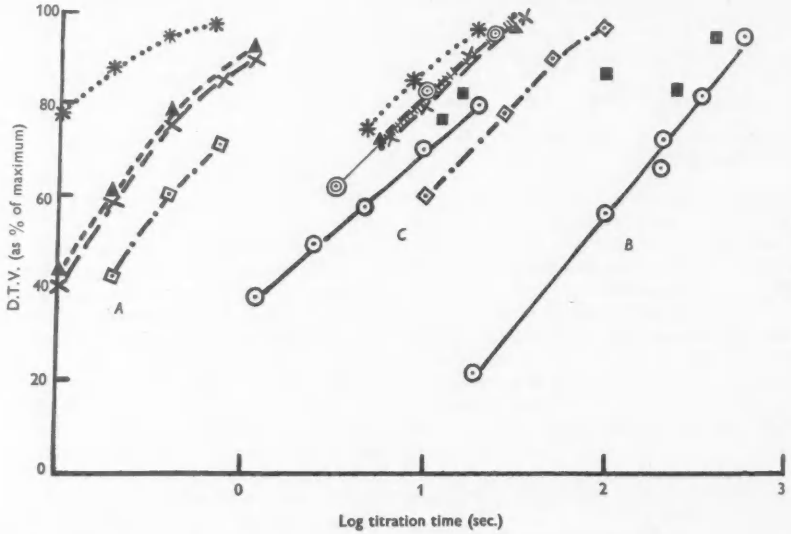

Fig. 4. Effect of titration time on dye titration value (D.T.V.) of ascorbic acid and allied compounds. Ascorbic acid, × — — ×; hydroxytetronic acid, ▲- - - -▲; dihydroxy maleic acid, ✳ • • • • ✳; reductic acid, ◇ — • — ◇; glucoreductone, ⊙——⊙; diketogulonic acid, ◎——◎; *iso*ascorbic acid, ● |||||| ●. Results obtained by Harris & Mapson (1947) on left-hand side (*A*), by Wokes (1946*b*) on right-hand side of diagram (*B*). Results of present work in centre (*C*). Results on walnut extracts indicated thus ■.

time was adequate. This method certainly corrects for some of the interfering substance, but the correction is not necessarily complete as, in the short but definite time required for the dye to react with the whole of the ascorbic acid, it may also have acted with some of the interfering substance. This was clearly shown by Harris & Mapson (1944, 1947) using the continuous flow method, with which they used reaction times down to 0·28 sec. They found that at pH 1·4 about 12% of ascorbic acid had not yet reacted with the dye 1·1 sec. after mixing, whereas about 40% of reductone had already reacted in this time under the same experimental conditions. They obtained velocity constants for reductic acid and for reductone which were re-

in the present paper. However, no indication has been obtained of the presence of *iso*ascorbic acid or of hydroxytetronic acid in walnut tissues. Wokes (1946*b*) described a modified potentiometric method with which reaction times were obtained to less than 10 sec. Using a titration pH of 0·6–0·7, he confirmed that *iso*ascorbic and hydroxytetronic acids reacted as quickly as ascorbic acid with the dye, and that dihydroxymaleic acid reacted still more rapidly. He also found that under the given conditions diketogulonic acid reacted as rapidly as ascorbic acid, reductic acid rather more slowly, and reductone much more slowly (see Fig. 4). Apparent vitamin C from different walnut tissues resembled reductone in reacting more slowly.

Spectroscopic studies

For these we used purified acid ethanolic extracts prepared, as described above, from different walnut tissues, but especially the buds and catkins, in which the dye titrations had shown the apparent vitamin C to represent practically the whole of the dye reductant. The extracts were examined using the

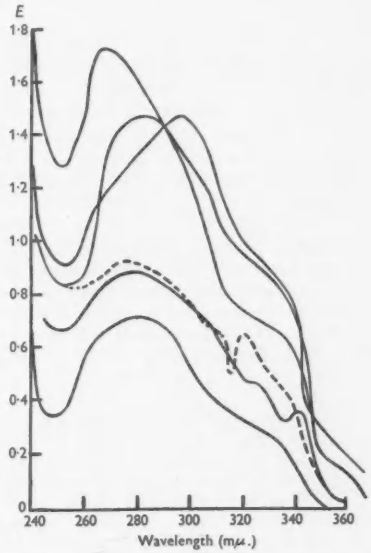

Fig. 5. Absorption curves of extracts of walnut buds and catkins, all in HPO_3 except the dotted curve which was obtained on an extract the pH of which had been adjusted to 4·5.

Hilger E 3 quartz spectrograph, and showed a broad absorption band with a main peak at 280–295 and indications of subsidiary peaks at 325–330 and 335–343 mμ. (Fig. 5). Except in one rather doubtful case, we were unable to obtain any evidence of the presence of reductone or of reductic acid, two of the possible constituents of apparent vitamin C which have been found in other plants.

DISCUSSION

The discovery in walnut tissues of extraordinarily high concentrations of apparent vitamin C raised interesting questions regarding its nature and possible functions. In earlier work we were guided by the hypothesis that the apparent vitamin C resembled that found in other foods and was probably an enediol of the reductone type. This hypothesis was supported by a close resemblance to reductone in its slow rate of reaction with the dye, a criterion which Harris & Mapson (1947) used to differentiate between various non-specific dye reductants. However, its failure to react with ascorbic acid oxidase, under conditions such that reductone would react, led us to doubt this hypothesis, and finally to abandon it on discovering (Wokes, 1946*a*) that, in contrast with reductone, the apparent vitamin C of walnuts showed a marked increase in dye titration value as the pH was reduced below 2. This was confirmed by our failure to detect reductone spectroscopically in numerous extracts of different walnut tissues, especially buds and catkins. The observation that a yellow colour developed when the apparent vitamin C was titrated with dye, or oxidized with the walnut enzymes, led us to investigate this oxidation product; and when we found that it had a similar spectrum to that of juglone, the possibility arose that some at least of the apparent vitamin C in walnuts is a reduced form of juglone. Although there was a high correlation between the increase in intensity of yellow colour and the concentration of apparent vitamin C in different tissues, occasional discrepant results suggested that in some instances at least there might be a second non-specific dye reductant present. Spectroscopic studies of numerous walnut extracts failed to identify the apparent vitamin C, the absorption curves showing evidence of fine structure which needed for its complete elucidation more accurate and more sensitive spectroscopic apparatus than was at our disposal.

The occurrence of apparent vitamin C in so many of the walnut tissues, especially those in which ascorbic acid is also found, suggests that it cannot be merely a waste product, but almost certainly plays an active part in some metabolic process, possibly one concerned with ascorbic acid. Had it proved to be reductone, as earlier postulated, it might then have been a precursor of ascorbic acid. Since reductone has been formed *in vitro* by the action of ultraviolet light on glyoxal, which is itself a possible product of photosynthesis, an attractive hypothesis could be developed for the photosynthesis of ascorbic acid from glyoxal via reductone. Probably in most plants synthesis of ascorbic acid occurs in green photosynthetic tissues, to be followed by translocation to the fruits. This could explain our failure to find apparent vitamin C in fruits or leaves of other plants. In the walnut, synthesis of ascorbic acid in the leaves might be incomplete, some of the reductone being translocated as such to the endocarp there to be converted into ascorbic acid. Such a picture would fit many observations, especially those concerning concentration gradients, which are more favourable to the translocation of apparent than of true vitamin C from leaf to fruit.

The above theory was discredited by our failure to obtain a significant increase in vitamin C concentration during the storage of immature fruits in which the concentration of apparent vitamin C was falling. It was abandoned when we obtained clear evidence that the apparent vitamin C is not reductone, but is more likely to be a juglone derivative, possibly a reduction product. Such a compound could hardly be a precursor of ascorbic acid, though it might be linked with the latter in an oxidation system. Further speculation must, however, be deferred until the chemical nature of apparent vitamin C has been more clearly established. Work on this problem is still in progress.

Since the above work was completed evidence has been obtained (Daglish & Wokes, 1948) that the apparent vitamin C in resting buds and catkins is mainly, if not entirely, a derivative of hydrojuglone (1:4:5-trihydroxynaphthalene).

SUMMARY

1. Apparent vitamin C has been detected in numerous samples from the walnut (*Juglans regia*).

2. In fruits the highest concentrations occurred in the epicarp and mesocarp, especially in very young fruits, in which it could form half of the total vitamin C, as measured by dye titration.

3. In leaves the highest concentrations occurred in the mesophyll, from which there was usually a definite concentration gradient through the vascular tissue, rachis and petiole towards the stem.

4. In the stem the highest concentration was in the phloem, in which the concentration gradient was generally from older to younger tissue during the early summer, but in the reverse direction in the winter.

5. Remarkably high concentrations of apparent vitamin C were found in buds and catkins, in which it usually formed most of the total vitamin C.

6. The rate of reaction of apparent vitamin C with the indophenol dye was found to be much slower than that of ascorbic acid, dihydroxymaleic acid, diketogulonic acid, hydroxytetronic acid or *iso*ascorbic acid, and to lie between those of reductic acid and reductone, all determined at the normal titration pH of about 0·7.

7. The apparent vitamin C in walnut tissues differs from reductone in (*a*) its stability towards ascorbic acid oxidase; (*b*) the increase of its dye titration value as the titration pH is reduced below 2; (*c*) the development, by the action of the indophenol dye or of the walnut enzymes, of a yellow compound which spectroscopically seemed to resemble juglone.

We are indebted to Prof. T. Reichstein for specimens of reductone and of reductic acid, to Dr F. Bergel for specimens of reductone, reductic acid, diketogulonic acid, hydroxytetronic acid and *iso*ascorbic acid, to the late Dr F. C. Jacoby for many of the spectroscopic estimations and to Miss J. G. Organ, Miss E. M. James and Miss E. Finnegan for assistance.

Note Since this paper was sent to the press we have been shown by Prof. Mirimanoff a thesis for Doctor of Pharmacy dealing with vitamin C in the walnut and confirming some of our findings. This thesis (Stadelmann, 1949) refers to work carried out in Germany during the war by J. Breinlich (Breinlich, 1942), in which the suggestion is put forward that the ascorbic acid in walnuts is oxidised to dehydroascorbic acid by juglone, which is itself reduced to hydrojuglone. No proof is provided of the existence of hydrojuglone in walnut tissues.

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Chemical Factors in the Germination of Spore-bearing Aerobes. The Effect of Yeast Extract on the Germination of *Bacillus anthracis* and its Replacement by Adenosine

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The rapidity of germination of bacterial spores when inoculated into a nutrient medium has been known for many years. Thus Fischoeder (1909) showed that 98% of anthrax spores became heat-sensitive in broth at 37° in 1 hr., but only 50% became heat-sensitive in saline at the same temperature in 5 hr. Little work has been done, however, on the specific nutrients responsible for this type of effect, in spite of advances in the study of the requirements for bacterial growth (reviewed by Knight, 1945). Thus the review of Knaysi (1948) on the bacterial endospore adds little on the conditions for germination to that of Cook (1932), apart from the work of Knaysi (1945) himself on *Bacillus mycoides* (*B. cereus* var. *mycoides*). Using a microscopic technique, he concluded that the spore is adequately supplied with stored nitrogen, but requires a source of carbohydrate and maintenance of a neutral pH. On the other hand, Keilin & Hartree (1947), using a metabolic method, found that *B. subtilis* required a source of assimilable nitrogen and an unidentified dialysable factor present in yeast extract, peptone or tryptic digest of casein.

More recently, Wynne & Foster (1948*a*, *b*, *c*), and Foster & Wynne (1948), have studied the subject with special reference to *Clostridium botulinum*. Their findings showed a need for carbon dioxide (Wynne & Foster, 1948*c*), and inhibition by traces of unsaturated fatty acids, recalling inhibition of the growth of non-sporing bacteria, e.g. *Mycobacterium tuberculosis* (Dubos, 1945) and *Haemophilus pertussis* (Pollock, 1947). In spite of the widespread occurrence of carbon dioxide requirements and unsaturated fatty acid inhibitions in bacterial nutrition, no such effects were observed (Wynne & Foster, 1948*c*; Foster & Wynne, 1948) with the germination of the four aerobes studied, *B. brevis*, *B. megatherium*, *B. mesentericus* (*B. pumilus*) and *B. subtilis*.

In other fields of biology, there has been a general interest in problems of dormancy, and it has been recognized that the germination of many dormant cells is stimulated by essential metabolites produced and secreted by active tissues (see Brown, 1946), and in some instances the active principles have been either identified chemically or replaced by pure chemical compounds.

The germination of the mould, *Phycomyces blakesleeanus*, was examined by Robbins and co-workers, who obtained two fractions from potato extract (Robbins & Hamner, 1940), both of which were essential for optimal activity. One of them could be replaced by guanine (Robbins & Kavanagh, 1942*a*) or by hypoxanthine (Robbins, Kavanagh & Kavanagh, 1942), and the latter was isolated from the extract by Robbins & Kavanagh (1942*b*). Other purines tested by Robbins (1943) were less active. The work of Hurni (1946) showed that the germination of *Rhizopus nodosus* was also stimulated by hypoxanthine, and that the early growth of four other Mucoraceae was similarly stimulated, though proof was not given that such stimulation was specifically connected with the phase of germination. With *Neurospora crassa*, on the other hand, the stimulating material in media containing autoclaved pentose was probably furfural (Emerson, 1948).

In a different field, the requirements for excystment of the ciliate protozoon, *Colpoda cucullus*, have been reported by Barker & Taylor (1933), Thimann & Barker (1934), Thimann & Haagen-Smit (1937), Haagen-Smit & Thimann (1938) and Prater & Haagen-Smit (1940). These authors found that, in addition to a source of soluble carbohydrate and various stimulating factors associated with carbohydrate metabolism, there was a need for one of two neutral compounds, $C_{13}H_{24}O_2$ or $C_{26}H_{44}O_2$, which were active at a concentration of 1 in $2{\cdot}5$–$5{\cdot}0 \times 10^7$. So far, it is not known if the nature of these compounds has been established, and their activity proved by the use of synthetic specimens.

It will be seen that in addition to unidentified factors, the compounds which have been implicated in the germination of dormant cells are of a very varied nature, no common pattern being yet discernible. The work now reported is concerned with the addition of results on the germination of spores of a single strain of *B. anthracis*. Results on other strains and species will be given in a later paper.

METHODS

The loss of resistance to heat, as a physiological criterion of germination shown by viable count, has been preferred in spite of the criticism (Cook, 1932;

Gardner, 1945) that this represents a pregerminative phase and does not correspond to true germination. The choice is justified, however, by the customary use of heat resistance in the diagnosis of spore formation and in freeing spore suspensions from vegetative cells. Support of this view has recently been elaborated by Wynne & Foster (1948*a*), and the evidence need not be repeated in detail. Briefly an organism, after loss of heat resistance, cannot be regarded as being in the spore state. A spore undergoing such a change must therefore be regarded as having germinated if still viable. The term 'germination' will therefore be used in this sense throughout.

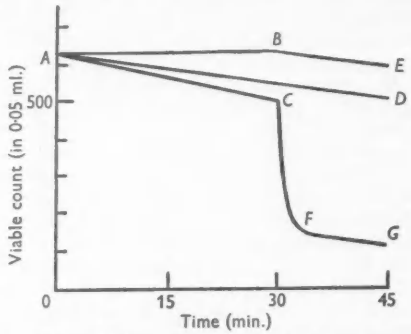

Fig. 1. Effect of time at different temperatures on viable count of *Bacillus anthracis* (diagrammatic). *AB* at 0° in nutrient medium; *AC* at 35° in nutrient medium; *AD*, *BE* and *CFG* at 60° in inert diluent.

General principle. This is shown in Fig. 1. If spores were suspended in a suitable culture medium, and dilutions in a non-nutrient but harmless fluid were made at intervals for determination of the viable count, the changes in count were either small or insignificant during a period of about 30 min. whether the culture medium was maintained at 0° (*AB*) or 35° (*AC*). If the dilutions were heated at 60°, little further change was shown over a period up to 60 min., either with those made initially (*AD*) or with those made from culture medium which had been maintained at 0° (*BE*). With dilutions from culture medium which had been maintained at 35°, on the other hand, the count fell rapidly (*CFG*), usually reaching a comparatively stable lower level in less than 5 min. The count of spores remaining resistant to heat at 60° after any treatment will be referred to as the S count, while counts set up without heating at 60°, including both resistant spores and non-resistant vegetative forms, will be referred to as the $S+V$ count. (These abbreviations are used instead of the terms 'spore count' and 'total count' to avoid confusion with the customary meaning of the latter in bacteriology.)

Spore suspensions. Spores of a virulent strain of *B. anthracis*, supplied by Dr R. L. Vollum, were dried in horse serum and stored *in vacuo*. Three different spore suspensions were prepared from this stock culture as follows: (*a*) Grown direct from the stock culture on the surface of CCY agar (Gladstone & Fildes, 1940) to about 50% sporulation in 2–3 days. (*b*) Grown from (*a*) to about 100% sporulation in 24 hr. in a vigorously aerated medium composed of 2% marmite, 1% cane molasses (decolorized with activated charcoal) and 0·5% $Na_2HPO_4.2H_2O$. The pH of this acid medium was adjusted to 7·3 with aseptic precautions after autoclaving. (*c*) Grown from (*a*) to about 90% sporulation in 48 hr. in the 'complete' synthetic medium of Gladstone (1939) with L-alanine in place of DL-alanine (Hills, 1949) and supplemented by 10^{-7} M-aneurin (Hagan, O'Kane & Young, 1943), 5×10^{-4} M-Ca^{++} (Brewer, McCullough, Mills, Roessler, Herbst & Howe, 1946), and 10^{-5} M-yeast adenylic acid. The inoculum was about 2000 spores/ml. (viable count), and the cultures were aerated by shaking at 120 oscillations/min. in 50 ml. conical flasks containing 10 ml. culture, the distance of travel being 5 cm. The strain was still virulent after growth on this synthetic medium (LD_{50} for mice, about 200 spores by the intraperitoneal route).

After reaping, all spore suspensions were heated 1 hr. at 60° to destroy remaining vegetative forms, washed thrice with distilled water and stored in the ice chest. Suspension (*a*) was used for the major part of the work, (*b*) was used only for a few preliminary experiments, and (*c*) was used after the discovery that a preparation of yeast adenylic acid promoted almost complete germination in a suitable synthetic medium. It was thus possible, by cultivation of spores in such a medium, to reduce transference with the inoculum of unknown biologically active metabolites. Without adenylic acid, the use of a synthetic medium involved cultures in which only a small proportion of the inoculum could have been shown to have germinated. Under these conditions, growth from those spores which germinated most easily may have produced a new population of spores with simpler requirements for germination than those of the original culture. Such suspensions were avoided.

Basal medium. One of the early difficulties was the design of a suitable deficient basal medium, since germination was too rapid for quantitative measurement in the usual laboratory media.

The medium finally adopted had the following composition: KH_2PO_4 (A.R., 4·5 g. in 450 ml. water); L-tyrosine (crude, see p. 357, 50 mg. in 25 ml. N-NaOH); HCl-hydrolysed gelatin (Nelson's photographic no. 1, neutralized, 12·5 ml. 20%). The constituents were made up together to 500 ml., brought to pH 7·4 with 1–2 ml. N-NaOH and autoclaved. This basal medium was double strength to allow for the addition of inoculum and various test preparations to a fixed total volume. Although not essential for germination, the following mixture, which was essential for good growth, was added aseptically just before use (0·5 ml./10 ml. double-strength medium): aneurin, 1 ml. 10^{-4} M; $MgSO_4$, 40 mg.; $FeSO_4.(NH_4)_2SO_4.6H_2O$, 16 mg.; $CaCl_2.6H_2O$, 110 mg.; glucose, 2·5 g.; in 5 ml. N-HCl with 20 ml. water; Seitz filtered. After this addition the medium was neutralized with 0·5 M-$NaHCO_3$ (0·2 ml./10 ml. double-strength medium) and distributed aseptically in accurately measured quantities in $6 \times \frac{3}{4}$ in. test tubes which had been cleaned in acid bichromate and sterilized with gauze-covered plugs.

In some experiments this basal medium was replaced by one containing 33 mM-phosphate buffer (prepared from

KH_2PO_4 (A.R.) with CO_2-free N-NaOH added to give pH 7·3).

Procedure. Before inoculation, the tubes of medium were warmed in a well-stirred water bath, thermostatically controlled at 35·0 ± 0·1°. No attempt was made either to aerate or to secure anaerobic conditions. To avoid errors due to the continuation of germination during the sampling of a long series of tubes, experiments were set up by inoculating at timed intervals, usually 1 min. Sampling, by making tenfold dilutions in distilled water, also took place at corresponding intervals. Where initial counts were required, samples were taken just after inoculation, and the tubes of inoculated medium were returned to the water bath immediately after sampling. For $S+V$ counts, the tubes of diluent were cooled in ice water before sampling and were kept in the cooling bath after sampling until the contents were plated. For S counts, the tubes of diluent were heated to 60° before sampling and heating at this temperature was continued for 15 min. after sampling to destroy all germinated forms. The dilutions were then cooled and kept in ice water till the time of plating.

Except where stated otherwise, the inoculum was about 12,000 spores/ml. and the period of incubation was 30 min.

Viable counts and their accuracy. The principle of the surface-plate technique of Miles & Misra (1938) was used with minor modifications in technical details. The composition of the medium for plating was 2% Evans peptone, 0·5% NaCl and 1·5% agar, pH 7·6. To ensure uniformity of successive batches of medium, it was made from a single batch of peptone, of which a sample had been shown to give satisfactory counts of the test organism, never significantly less, and, with some suspensions, many times greater than those on tryptic ox agar. Plates were poured without cooling the melted agar and were dried, inverted over the lids, for 90 min. at 60°. Dropping pipettes, delivering 42 drops/ml., were used to distribute 7 drops of appropriate dilutions over each of 3 plates. Plates were incubated either for 12 hr. at 37° or for 15 hr. at 30°, when the colonies were discrete and of uniform size, about 1 mm. in diameter, provided that the number on each plate did not exceed 250. In designing the experiments, control plates were inoculated so as to yield about 200 colonies from the spores which had failed to germinate after incubation in the deficient basal medium. With the usual inoculum, this was attained by plating, as described, 0·5 ml. of the tenfold dilution made in sampling. Samples showing a fall in count due to germination were plated at the same dilution.

Ideally, the chance of observing a particular value in a series of replicates should conform to a Poisson distribution, for which both the mean and the variance are estimated by the mean of the observations (Fisher, Thornton & Mackenzie, 1922). For a total control count of 500–700 colonies on the three plates, the standard error was thus about 25 colonies and the limits were of the order of ±8% with a probability, $P=0{\cdot}95$. Ultimately, there were available for testing conformation to a Poisson series, 47 sets of quadruplicate controls and 21 sets of quintuplicates. With such a small series, it was necessary to group the data at both tails of the distribution, each amounting to 10% of the whole, to avoid expectations less than 5 in any group (Table 1). Owing to the presence of four aberrant values exceeding the theoretical 1% level, the χ^2 for deviations from the theoretical distribution could not therefore accurately summarize the whole of the available information. It indicated a larger probability, 0·5, for the occurrence of greater deviations by chance than 0·18 shown by the total χ^2 for variations within sets of replicates, the latter measure being very sensitive to the presence of a few excessively variable replicates, while giving little information on the general form of the distribution. Taking both criteria into consideration, it was considered justifiable to regard this sample of counts as conforming to a Poisson series, but in using the square root of a count as an estimate of its standard deviation, in order to assess the significance of differences, the precaution was

Table 1. *Conformation of control counts to Poisson series*

Probability of observing greater deviations by chance	No. of sets of replicates observed: Sets of 4	Sets of 5	Total n	No. of sets expected (N)	Difference ($n-N$)	$\frac{(n-N)^2}{N}$
1·00						
0·99	0	0	0			
0·98	1	0	1	6·8	+2·2	0·71
0·95	2	2	4			
0·90	1	3	4			
0·80	5	2	7	6·8	+0·2	0·01
0·70	6	2	8	6·8	+1·2	0·21
0·50	6	5	11	13·6	−2·6	0·50
0·30	6	2	8	13·6	−5·6	2·30
0·20	8	3	11	6·8	+4·2	2·58
0·10	6	1	7	6·8	+0·2	0·01
0·05	1	0	1			
0·02	1	0	1	6·8	+0·2	0·01
0·01	1	0	1			
0·00	3	1	4			
Total	47	21	68	68·0	0·0	6·33
Total χ^2 for deviations of counts from mean of set	175·4	69·3	244·7	—	—	—
Degrees of freedom (ϕ)	141	84	225	—	—	7
$\sqrt{2\chi^2}-\sqrt{(2\phi-1)}$	1·97	−1·15	0·93	—	—	—
Probability of observing greater deviations by chance	0·025	0·88	0·18	—	—	0·5

adopted of examining the data in a particular experiment for evidence of excessive variation.

Comparison of potencies of active preparations. The effects of preparations promoting the germination of spores in the presence of the basal medium were measured by the decrease in *S* count caused by the preparation in excess of the small decrease observed in the basal medium alone under the same conditions.

The control *S* count, after incubation in the basal medium, was used as an estimate of the number of spores which had been subject to the influence of the active preparations.

For comparison of potencies it was found that a convenient linear concentration relationship could be obtained by the probit-log concentration transformation (see Finney, 1947). Statistical justification of this procedure will be published elsewhere. Simple graphical methods sufficed, however, for general use.

RESULTS

Preliminary experiments

The rate of germination of a small inoculum, about 1000 spores/ml., in the usual laboratory media, was so rapid that a high proportion of spores lost their resistance to heat during the time required to make the inoculation and take a sample. On the other hand, the rate was so slow in the synthetic medium of Gladstone (1939), that it was not measurable by the technique adopted. The proportion of spores which became sensitive to heat in a complex medium during a convenient experimental period could be controlled, however, by increasing the size of the inoculum, or by diluting the medium. The latter method was chosen in the first place, in order to keep the inoculum size as small as possible, and so avoid transferring to fresh medium unknown essential metabolites present in the seed. It is recognized that this could be no more than a temporary expedient, since investigation of the effect of a particular nutrient might be of little value if concurrent deficiencies of other nutrients occurred.

In order to gain some idea of the types of material essential for germination, the CCY medium of Gladstone & Fildes (1940) was used since its composition, though complex, can be subjected to some degree of controlled variation. The bulk of the amino-acids were supplied by an acid hydrolysate of casein, while those destroyed by acid hydrolysis, especially tryptophan, were supplied by a tryptic digest of the same protein. Other growth factors were supplied by yeast extract. Using this medium at 10% of the usual concentration (Table 2), it was shown that the tryptic digest was not essential, and that the small activity of the acid hydrolysate (Exp. 1), with or without tryptic digest, was much enhanced by yeast, although the latter, in the presence of buffer, was inactive (Exp. 2). The yeast extract was not replaceable by a mixture of known growth factors. Its fractionation was therefore undertaken with the object of identifying the active components.

Table 2. *The effect of constituents of CCY medium on germination*

(Basal medium, 0·05M-glycerophosphate, pH 7·3. The additions were prepared as described by Gladstone & Fildes (1940) and the final concentrations in the medium were acid-hydrolysed casein (*AC*), 0·07%; tryptic digest of casein (*TC*), 0·03%; yeast extract (*Y*) equivalent to 0·2% fresh yeast.)

Exp. no.	Additions to basal medium	Spore count at 0 min.	15 min.	30 min.	60 min.
1	None	446	—	—	362
	AC	447	462	376	198
	AC+*TC*	509	478	382	208
	AC+*Y*	340	140	58	30
	AC+*TC*+*Y*	483	131	74	29
2	None	608	—	520	—
	Y	571	—	562	—
	AC	646	—	372	—
	AC+*Y*	615	—	48	—

The diluted casein hydrolysate did not, however, provide a suitable basal medium for the assay of yeast fractions, since the degree of germination, even in the absence of yeast extract, was too high except at dilutions which may well have been suboptimal in several essential metabolites. The synthetic medium of Gladstone (1939) was also unsuitable, since, as in buffer, the activity of yeast was not observed in this medium. It became necessary, therefore, to devise a suitable basal medium.

Development of a basal medium for assay of yeast fractions

A suitable basal medium should, in general: (i) contain optimal amounts of all essential nutrients except that to be assayed, and (ii) usually be as free from the material to be assayed as possible. Condition (i) is satisfied when the biological effect in the presence of an active supplement is independent of the concentration of the basal medium within suitable limits. Condition (ii) is satisfied when the effect in the basal medium alone is small.

After a number of trials, a hydrochloric acid hydrolysate of gelatin, supplemented by tyrosine and buffered by 33 mM-phosphate, pH 7·3, was found to allow a satisfactory response to yeast extract. In the presence of 0·28 mM-L-tyrosine, the optimum concentration of gelatin hydrolysate was 0·25% (Fig. 2). Lower concentrations of gelatin were little less effective, but higher concentrations were inhibitory. No advantage in this respect was offered by a hydrolysate of lower salt content made with sulphuric acid and treated with barium hydroxide to remove sulphate ions. A fivefold increase in the tyrosine concentration, supplied either as the pure isomer or as twice the amount of synthetic DL-tyrosine, did not increase the extent of germination (Table 3). The initial counts showed that the presence

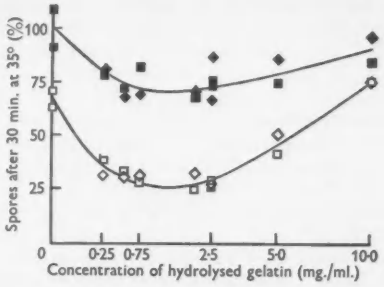

Fig. 2. Optimum concentration of hydrolysed gelatin for germination in presence of 0·005% tyrosine. *A*, basal medium with gelatin hydrolysed by 23% HCl, 0·75 hr. at 120°, ■; *B*, basal medium with gelatin hydrolysed by 10% H_2SO_4, 3 hr. at 130°, ◆; *A* + yeast extract equivalent to 0·2% fresh yeast, □; *B* + yeast extract equivalent to 0·2% fresh yeast, ◇. To increase the range covered, the horizontal scale is proportional to the square root of the concentration.

of tyrosine in the dilutions during heating did not significantly affect the capacity of the spores to develop colonies on plating. For routine use, a crude sample of L-tyrosine, obtained as a by-product from the preparation of tryptic digest of casein, was found to be as suitable as the pure preparations.

Table 3. *Effect of tyrosine on germination in presence of acid-hydrolysed gelatin and yeast extract*

(The basal medium contained HCl-hydrolysed gelatin, equivalent to 0·25% (w/v) gelatin, and yeast extract equivalent to 0·02% (w/v) fresh yeast in 33 mM-phosphate buffer, pH 7·3.)

Addition to basal medium	Concn. of each enantiomorph (mM)	Spore count at 0 min.	Spore count at 30 min.
None	—	375	283
L-Tyrosine	0·28	381	106
	1·4	369	89
DL-Tyrosine	0·28	384	99
	1·4	405	103

Table 4. *Effects of various metabolites on germination in acid-hydrolysed gelatin and tyrosine, with and without yeast extract*

(Basal medium, HCl-hydrolysed gelatin equivalent to 0·25% gelatin, 0·28 mM-L-tyrosine (crude) in 33 mM-phosphate buffer pH 7·3.)

Additions to basal medium	Concn. (M)
Cations	
Ca^{++}	$1·0 \times 10^{-3}$
Fe^{++}	$0·8 \times 10^{-4}$
Mg^{++}	$0·5 \times 10^{-3}$
Glucose	$1·3 \times 10^{-2}$
Guanine and pyrimidines	
Cytosine	$4·5 \times 10^{-5}$
Guanine	$3·3 \times 10^{-5}$
Thymine	$4·0 \times 10^{-5}$
Uracil	$4·5 \times 10^{-5}$
Sulphur compounds	
Cystine (L-)	$1·0 \times 10^{-4}$
Glutathione	$1·0 \times 10^{-3}$
Tryptophan (L-)	$0·5 \times 10^{-6}$
Vitamins	
p-Aminobenzoate	$0·8 \times 10^{-5}$
Aneurin	$2·0 \times 10^{-6}$
Ascorbic acid	$0·4 \times 10^{-3}$
Choline	$0·4 \times 10^{-5}$
Inositol	$0·4 \times 10^{-3}$
Nicotinic acid	$0·7 \times 10^{-5}$
Pantothenate	$2·0 \times 10^{-6}$
Pimelate	$0·7 \times 10^{-4}$
Pyridoxine	$0·8 \times 10^{-5}$
Riboflavin	$2·0 \times 10^{-6}$

(Since the technique gave control counts conforming to the Poisson distribution (Table 1), the standard error (s) of the difference between two counts (D) could be taken as the square root of the sum of the two counts. Ratios D/s exceeding 1·96 were considered to show significant differences ($P = 0·95$) and are given in heavy type.)

Additions to basal medium	Initial spore count	D/s	After 30 min. at 35°: No yeast — Spore count	D/s	After 30 min. at 35°: 0·03% yeast — Spore count	D/s
None	644	—	641	—	253	—
Cations	651	0·19	**529**	**3·28**	243	0·45
Glucose	638	0·17	593	1·37	296	1·84
Guanine and pyrimidines	684	1·10	648	0·19	**348**	**3·88**
Sulphur compounds	684	1·10	627	0·39	234	0·86
Tryptophan	642	0·06	584	1·63	247	0·27
Vitamins	665	0·59	605	1·02	286	1·42
All	679	0·96	638	0·08	**303**	**2·13**

In order to check the adequacy of the supply of essential factors in this basal medium, the effects of various known growth factors were tested, not only on the blank, but also on the germination with suboptimal amounts of yeast extract (Table 4). The materials tested included cations, glucose, guanine, pyrimidines, sources of organic sulphur, tryptophan and water-soluble vitamins. No pronounced effects were observed, but those which were statistically significant were the small inhibition of 15% by guanine plus pyrimidines in the presence of yeast, and the small promotion of germination of 14% by cations in the absence of yeast. These effects could have no influence on the assay of yeast fractions, since the concentrations, at levels similar to those found optimal for growth by Brewer *et al.* (1946), were at least 50-fold greater than those likely to be present in yeast extracts of much greater activity. The cations, however, together with aneurin and glucose, were included in the basal medium, since they were essential for good growth and therefore, presumably, for survival of germinated forms. Details for preparation of the medium are given in the section on Methods, p. 354.

Replacement of yeast factor by adenine derivatives

After the development of a suitable basal medium, the problem was pursued along two independent lines. Boiled yeast extracts, or boiled autolysates up to twenty times as active as such simple extracts, were fractionated to give preparations active at 2·5–5·0 μg./ml. These experiments are not described in detail, since the testing of materials known to be present in yeast extract showed activity in preparations of many nucleic acid derivatives (Table 5).

A commercial sample of yeast adenylic acid was highly active, and a guanylic acid preparation also showed some activity, but adenine and guanine were inactive. Preparations of yeast and thymus nucleic acids also showed some activity. In a second experiment (Table 5) the purines were tested with and without ribose to see if the latter made the synthesis of an active compound possible. Significant effects were not observed. Fig. 3 shows that the maximum

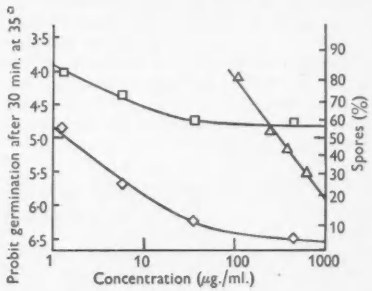

Fig. 3. Effect of nucleotide preparations and yeast extract on germination. Adenylic acid, ◇; guanylic acid, □; yeast extract, △.

effect was given by both adenylic acid and guanylic acid at a concentration of about 35 μg./ml. or 10^{-4}M, but the latter showed only 40% germination, compared with over 90% for adenylic acid. The effect of adenylic acid, however, was qualitatively different from that of yeast, since twofold increase of yeast concentration increased germination from 45 to 70%, whereas fivefold increase in adenylic acid concentration was necessary to produce a similar change in germination.

It must be emphasized that the preparations used up to this stage had been commercial products which had not been specially purified. The activity may therefore have been due to impurities. This was also suggested by the properties of the material in yeast extract, in particular the failure to get a barium salt insoluble in 67% ethanol. Further, during the course of work on the amino-acids essential for germination (Hills, 1949), it was found that there was a five- to ten-fold increase in the activity of the sample of adenylic acid used (Table 6) in 5 mM-aqueous solution at its own pH (3·1) during a period of about 6 months at room temperature. In view of the probability of hydrolysis under these conditions, a commercial sample of adenosine was tested at the same time and found to have practically the same

Table 5. *Effect of nucleic acid derivatives on germination in basal medium*

(Ribose, where added, was 4×10^{-4}M. Other additions were $2{\cdot}5 \times 10^{-4}$M, except yeast adenylic acid, 10^{-5}M, guanylic acid, 10^{-4}M and yeast extract equivalent to 375 μg. fresh yeast/ml. (Exp. 1) or 500 μg./ml. (Exp. 2).)

Exp. no.	Additions to basal medium	Spore count after 30 min. at 35°	
		Without ribose	With ribose
1	None	495	
	None	491	
	None	463	
	Adenine	492	
	Adenosine triphosphate	550	
	Adenylic acid	216	
	Guanine	490	
	Guanylic acid	302	
	Thymus nucleic acid	434	
	Yeast nucleic acid	265	
	Yeast extract	201	
2	None	724	766
	None	938	938
	Adenine	908	944
	Guanine	757	948
	Hypoxanthine	764	944
	Xanthine	892	826
	Adenylic acid	126	67
	Yeast extract	122	102

activity as the old solution of adenylic acid, i.e. five to seven times the activity of freshly made solutions of preparations of yeast adenylic acid from two different commercial sources. Adenine cytosine dinucleotide had the same activity as adenylic acid on a molecular basis, but a guanosine preparation showed much smaller activity.

Table 6. *Concentrations of preparations of nucleosides and nucleotides for 50 % germination under standard conditions*

(The conditions were 30 min. at 35° in the basal medium described on p. 354. The concentration for a final spore count 50% of that in the basal medium was estimated from points in the neighbourhood of 50% survivors on the assumption that the probit-log concentration regression line was parallel to that of the standard yeast preparation, which gave 50% survivors at 131–151 μg./ml. in these experiments.)

Addition to basal medium	Concn. for 50% germination (μM)
Adenosine	0·8
Yeast adenylic acid	
Sample *A*, fresh solution	4·0
Sample *A*, 6-month-old solution	0·5
Sample *B*, fresh solution	5·5
Adenine cytosine dinucleotide	6·1
Guanosine	80

In view of the importance of tyrosine in germination and the similarity in the stability of tyrosine codecarboxylase (Gale & Epps, 1944) to the germination factor from yeast, it seemed likely that this coenzyme might be a possible biologically active impurity in commercial preparations of nucleic acid derivatives. A sample, kindly supplied by Dr E. F. Gale, was, however, found to be inactive even at 1 mg./ml.

From these observations it appears that the activities of nucleic acid derivatives might reasonably be accounted for by their content of adenosine, a preparation of which was the most active material tested.

Purification of adenosine

In order to complete the identification of adenosine as the active material, the changes in activity were examined (Table 7), following purification by recrystallizations from water without change in m.p. 224–226° (uncorr.). The final product, dried *in vacuo* over $CaCl_2$, gave the following analytical results (Weiler and Strauss): (Found, C, 44·4; H, 5·2; N, 25·6. Calc. for $C_{10}H_{13}N_5O_4$; C, 44·9; H, 4·9; N, 26·2 %). Levene & Bass (1931) gave m.p. 229°, with 1·5 mol. water but the conditions of drying were not stated. Fractions *D* and *E*, the mother liquor and crystals, respectively, from the third recrystallization, had closely similar activities at all four levels tested. The original commercial specimen *A* and the mother liquors *B* and *C*, from the first and second recrystallizations, respectively, were less active, except for the two lowest levels of preparation *C* in Exp. 1. The χ^2 test was therefore applied on the assumption that the best estimate for each of the levels tested was the mean of the corresponding values for *D* and *E*. Highly significant differences ($P < 0{\cdot}001$) were observed for *A*, *B* and *C* in both experiments, but the deviations for *D* and *E* together were not significant ($P = 0{\cdot}21$).

Sterilization of adenosine

In the experiments reported so far, the adenosine had been sterilized by autoclaving in aqueous solution (15 lb./in.²; 20 min.). The purest preparation *E*

Table 7. *Effect of recrystallization on activity of commercial sample of adenosine in promoting germination*

(The significance of differences was tested by χ^2 for the deviations of all points for each preparation from the means of corresponding points for preparations, *D* and *E*. The control counts, without addition of any preparation, were 668·4 and 597·8 in Exps. 1 and 2 respectively, each the mean of quintuplicates.)

		Spore count after 30 min. in basal medium + preparation				
		A	*B*	*C*	*D*	*E*
			Mother liquors from			
Exp. no.	Adenosine concn. (μg./ml.)	Original sample	1st recrystallization	2nd recrystallization	3rd recrystallization	Solid phase from 3rd recrystallization
1	0·188	537	558	515	523	515
	0·470	425	426	334	370	330
	1·175	318	278	301	267	239
	2·94	163	191	193	151	160
2	0·188	528	608	637	393	397
	0·470	362	386	401	304	331
	1·175	243	290	175	132	164
	2·94	156	157	148	114	130
χ^2		153·8	303·7	296·6	10·76	
Probability of difference due to chance		0·001	0·001	0·001	0·21	

was tested after sterilization both by autoclaving and by Seitz filtration (Table 8). Exp. 1 showed no significant differences at any concentration, and although Exp. 2 suggested that the filtered solution was less active at each concentration tested, only at the highest concentration did the difference appear to be significant by a simple χ^2 test. Examination of replicate counts in this experiment showed greater variability than usual ($P = 0{\cdot}14$), especially for those counts showing the apparently significant difference between autoclaved and filtered solutions. Partition of χ^2 in the form of an analysis of variance and application of a variance ratio test showed that the difference was not, in fact, significant ($P = 0{\cdot}09$). The conclusion is that if any loss of activity occurred on autoclaving it must be equal to the loss by adsorption on Seitz filtration. There is no evidence that the activity of adenosine was due to breakdown on autoclaving.

Effect of adenosine on vegetative and spore counts

Except for a few early experiments in complex media it has been assumed throughout this work that the fall in spore count on incubation in culture media was due to germination. Omission of controls on possible changes in the total count was justified in the assay of preparations, since the observation of such controls would have doubled the experimental work, complicated the computations of activity and the errors of random sampling would have reduced the value of any adjustments which such changes might have made in the probit. Table 9 shows that the effect of adenosine was solely on the loss of heat resistance of spores; the 30 % fall of the $V+S$ count on incubation was independent of the addition of adenosine to the basal medium, and whilst the proportion of heat-resistant organisms remained

Table 8. *Effect of purified adenosine on germination after sterilization by autoclaving or Seitz filtration*

(Thrice recrystallized adenosine was used (Preparation *E*, p. 359). Sterilization was carried out in 2 mM-solution; autoclaving for 20 min. at 15 lb./in.^{2}, and Seitz filtration through a Ford Sterimat S.B., 10 ml. through a pad 3·6 cm. in diameter. Control spore counts with no adenosine in the basal medium were 392·6 and 922·2 for Exps. 1 and 2 respectively (sum of three plates, each sum the mean of quintuplicate determinations). The period of incubation was 30 min. at 35°.)

		Spore counts with adenosine						
		Autoclaved		Filtered				
Exp. no.	Concn. of adenosine (μM)	Single plates	Sum (n_1)	Single plates	Sum (n_2)	$\frac{\chi^2}{(n_1-n_2)^2}{n_1+n_2}$	Degrees of freedom (ϕ)	Probability of greater difference due to chance (P)
1	0·6	81	289	82	264	1·13	1	—
		99		89				
		109		93				
	1·8	49	159	56	179	1·18	1	—
		52		61				
		58		62				
	6·0	40	123	37	117	0·15	1	—
		41		39				
		42		41				
2	0·6	219	692	235	745	1·95	1	—
		231		241				
		242		269				
	1·8	133	450	150	490	1·70	1	—
		148		152				
		169		188				
	6·0	60	211	65	258	4·71	1	0·03
		74		89				
		77		104				
						10·82	6	0·09
		1924		2053		4·18	1	0·04

Analysis of variance:

	ϕ	χ^2	F	P
Treatments	1	4·18	3·20	0·09
Experiments	1	3·77	2·89	>0·1
Concentrations	2	2·35	0·90	>0·1
Interaction	2	0·52	0·20	>0·1
		10·82		
Plates	24	31·38		

practically 100% after incubation in the basal medium, it fell to 11% in the basal medium plus 2 μM-adenosine. The remaining 89%, which were no longer spores since they had become heat sensitive, must be regarded as having germinated since they were still viable, giving visible colonies on plates of a suitable nutrient medium. Adenosine alone was ineffective in the absence of the basal medium.

Table 9. *Effect of adenosine on vegetative and spore counts*

(Sample equivalent to 0·05 ml. undiluted culture.)

	Count of sample			
	Before incubation		After 30 min. at 35°	
Medium	Vegetative + spore	Spore	Vegetative + spore	Spore
Basal	688	680	498	492
Buffer + 2 μM-adenosine	702	694	560	467
Basal + 2 μM-adenosine	719	694	472	52

DISCUSSION

The importance of nucleic acid derivatives in bacterial nutrition was first shown by Richardson (1936), when he reported the need of *Staphylococcus aureus* for uracil during anaerobic growth, although the organism was capable of synthesizing this essential pyrimidine aerobically. Subsequent workers have established the need for nucleic acid derivatives by other organisms. In some cases, complex derivatives have been more effective than simple purines and pyrimidines, e.g. with *Neurospora* mutants (Loring & Pierce, 1944) or *Lactobacillus gayonii* (*L. fermenti*) (Hutchings, Sloane & Boggiano, 1946).

The mode of action of adenosine in germination, however, is at present obscure, since nothing is known either of the biochemical or biophysical changes which occur in the spore during germination, or of the metabolism of adenosine by the spore. Adenosine can be hydrolysed and deaminated by various non-sporing organisms (Lutwak-Mann, 1936) and can stimulate the deamination of adenine by *Escherichia coli* (Stephenson & Trim, 1938) or of serine by *Clostridium welchii* (Woods & Trim, 1942). It would be of interest to examine the extent to which spores may catalyse such reactions as well as those of the type, purine-riboside + phosphate ⇌ purine + ribose-1-phosphate, described in animal tissues (Kalckar, 1945). Such processes involving ammonia and phosphate transfer might provide a link between the stimulation of anthrax spores by adenosine and that of Phycomyces spores by guanine or hypoxanthine (Robbins & Kavanagh, 1942*a*, *b*).

A distinction must be drawn between the requirements for germination and those for growth. The synthetic ability of cells emerging from a dormant state may be less than those of an actively growing culture (cf. Brown, 1946). The requirements for germination would thus tend to be more exacting than those for growth, as shown by the stimulation of germination but not of growth, by adenosine in the case of *B. anthracis*. In germination, with changes in a single cell without multiplication, it may be more difficult to show a requirement for a particular nutrient, since the cell may carry a supply, though insufficient for optimal activity, and the amount available for each cell cannot be markedly reduced as during growth. This may be the case with pyridoxal phosphate, an important coenzyme in metabolism of amino-acids, including that of tyrosine (see Gale, 1946); however, although tyrosine was important in the germination of *B. anthracis* even high concentrations of the coenzyme had no influence. In growth, involving perhaps a million-fold increase in cell substance, provided that the cell is sufficiently equipped with essential metabolites for initiation of the process, the increased synthetic ability of the actively metabolizing culture may be capable of supplying essential materials which could not be synthesized by the germinating spore. In this way it is possible to explain the growth of *B. anthracis* in synthetic media not containing adenosine (Gladstone, 1939; Brewer *et al.* 1946). In such media growth is due to the germination of only a small proportion of the inoculum in the early stages, presumably under the influence of traces of adenosine in the cells. This proportion is frequently not detectable by the comparatively crude viable-count technique. Increased germination cannot be excluded after changes have been produced in the medium by the actively growing culture, but it is masked by formation of fresh spores.

Although adenosine was the most active material tested in a high degree of purity, commercial preparations of yeast nucleic acid and its derivatives showed some activity. These materials were not purified to show how far the activity was specific for adenosine and how far it was due to related compounds. Adenine, however, was inactive, so that the specificity was greater than that of the Neurospora mutant described by Pierce & Loring (1945), for which the maximum growth rate was largely dependent on the amount of adenyl radical available rather than its state of combination. In view of the high sensitivity of the germination of *B. anthracis* to adenosine, this process might be made the basis of a microbiological assay. Less than 0·5 μg. could be detected and a three-point assay could be carried out on as little as 2 μg. without modifying the experimental technique already used. Since only 1% of the actual sample available was used in plating, the method could be made even more sensitive by the use of micropipettes. Of established

methods at present available, the guinea-pig auricle method of Drury, Lutwak-Mann & Solandt (1937) requires 1–2 μg., while the use of a specific deaminase required about 1 mg. unless the reaction is followed by ultraviolet spectrometry (Kalckar, 1947) when the amount may be reduced to 0·1 μg. The results given in Table 6 suggest an adenosine content of about 150 mg./100 g. fresh yeast for the best yeast extract used in this work. Ostern, Terszakowec & Hubl (1938), using the deaminase method, found 360 mg./100 g. dialysed acetone-dried yeast after 20 hr. autolysis, and this was equivalent to 60 % of the adenine of the original cell nucleic acid. The amount found by the effect on germination therefore does not exceed the maximum expected from the work of Ostern *et al.* (1938), but a closer comparison is not possible on account of the effects of autolysis and other conditions.

SUMMARY

1. The metabolites essential for the rapid germination of spores of *Bacillus anthracis* have been studied, using loss of resistance to heat as a measure of germination, determined by viable count.

2. A suitable deficient medium for the assay of promoters of germination contained 0·25 % acid-hydrolysed gelatin and 0·005 % L-tyrosine. Higher concentrations of hydrolysed gelatin were inhibitory.

3. The slow germination in the basal medium was accelerated significantly by yeast extract equivalent to 0·01 % fresh yeast.

4. The yeast extract was replaceable by a purified preparation of adenosine which gave a significant effect at a concentration of 0·2 μg./ml.

I have great pleasure in thanking Dr D. W. Henderson and Dr D. D. Woods for careful criticism of this paper. The experimental work was carried out, with the technical assistance of Cpl. W. Bailey, R.A.M.C., while the author was a member of the Scientific Staff, Medical Research Council. Permission to publish has been granted by the Chief Scientist, Ministry of Supply.

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Chemical Factors in the Germination of Spore-bearing Aerobes. The Effects of Amino-acids on the Germination of *Bacillus anthracis*, with some Observations on the Relation of Optical Form to Biological Activity

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The first paper of this series (Hills, 1949) has shown that, in a basal medium in which essential amino-acids are supplied by a gelatin hydrolysate and tyrosine, the rate of germination of spores of *Bacillus anthracis* was greatly increased by boiled yeast extract and that the extract could be replaced by adenosine at a concentration of 10^{-6}M or about 0·25 mg./l. It thus became possible to study the effects of amino-acids on germination, since the optimum concentration expected for many of them, of the order of 100 mg./l. on the basis of the optimum concentration of gelatin hydrolysate (0·25%), was much greater than that of the adenosine. Observations on amino-acid requirements were therefore not likely to be vitiated by trace impurities in the adenosine, which was, necessarily, a natural product.

Difficulty was encountered in the study of amino-acid requirements, however, since the synthetic medium for the growth of *B. anthracis* used by Gladstone (1939), and since modified by Brewer, McCullough, Mills, Roessler, Herbst & Howe (1946) supported a rate of germination, even in the presence of yeast extract or adenosine, too small for detection by viable counts, though some germination must have occurred since the strain used grew satisfactorily on the modification of the latter authors. Gladstone (1939) had shown, however, that growth in a modification of his medium containing fourteen amino-acids was inhibited by leucine, isoleucine and valine separately, but was stimulated when these three amino-acids were present in appropriate proportions. In a further simplified medium, containing a total of eleven amino-acids, these three were essential. It seemed possible, therefore, that the feeble germination in synthetic media, and the inhibition of germination which occurred with concentrations of gelatin hydrolysate above the optimal (Hills, 1949), might both be due to an unsuitable balance of amino-acids. Work was therefore begun by attempting to simulate the effect of excess gelatin hydrolysate by means of amino-acids, with the hope that this would reveal the cause of failure to germinate in the amino-acid medium.

METHODS

Technique. Experimental methods, based on the determination of viable counts by the surface plate technique of Miles & Misra (1938), were as described previously (Hills, 1949). It is again emphasized that all counts were viable counts and the abbreviation ($S+V$ count) is used for the sake of brevity to denote a count determined so as to include both spore and vegetative cells. The period of incubation was 30 min. at 35°, except where otherwise stated.

Materials. These were largely of commercial origin, but the adenosine and inorganic constituents of the media were purified by recrystallization thrice from water. One specimen of D-alanine was kindly presented by Roche Products Ltd. A second specimen, for which the author thanks Dr H. N. Rydon, was prepared by resolution of the benzoyl derivative of commercial synthetic DL-alanine with brucine, and had $[\alpha]_D^{22°} = -7{\cdot}8°$ at c, 1·342 g. hydrochloride/100 ml. N-HCl, indicating 90% optical purity. A specimen of L-alanine, also prepared by Dr Rydon by resolution of the benzoyl derivative with strychnine, was optically pure, having $[\alpha]_D^{21°} = +10{\cdot}7$ at c, 1·684 g. hydrochloride/100 ml. N-HCl.

The author thanks Dr Rydon also for a specimen of synthetic DL-tyrosine.

Pyruvic acid was purified by vacuum distillation and stored in the cold as 5M-solution (Wendel, 1932). Lactate was purified by recrystallization of the Zn salt and Zn was removed by Na_2CO_3.

Concentrations of materials have been expressed as the molarity of a single enantiomorph; the total concentration of a DL-mixture was thus twice that given.

Statistical interpretation of results. Since the technique was shown in the previous paper to give counts conforming to Poisson distributions, it was possible to take each count as an estimate of its sampling variance, and the sampling variance of the difference between two counts at the same dilution was therefore equal to the sum of the counts. This simple technique sufficed for the assessment of the differences between counts in the same experiment by well-known methods as described, for example, by Fisher (1946) or Davies (1947).

In comparing the proportions germinating in separate experiments it is necessary to calculate the variance of the proportion. This is not given by the usual formula based on the binomial distribution, since the observations are not the numbers germinating and failing to germinate, but are estimates n of the spores subjected to a particular treatment

and s of those failing to germinate, both determined on equal portions. Since these vary independently according to Poisson distributions, the sampling variance of $q\ (=s/n)$, the proportion of spores not germinating, is given by

$$V(q)=(\partial q/\partial s)^2\,V(s)+(\partial q/\partial n)^2\,V(n).$$

If n is the mean of k replicates and s that of l replicates

$$V(n)=n/k;\quad V(s)=s/l$$

and $$V(q)=(s/l+s^2/kn)/n^2 \quad \text{or} \quad (q/l+q^2/k)/n.$$

The second term of these expressions for $V(q)$ is usually small, contributing less than 10% to the standard error if $k=5l$. This frequently arises, since the initial counts for a number of independent treatments rarely differ significantly, and may therefore be pooled to give the best estimate of n.

In studying the effects of amino-acids as inhibitors of germination (Table 8), uncontrollable variations in conditions led to variations in the degree of germination of the uninhibited control in the different experiments. It was then found advantageous to normalize the data by transformation of proportions to probits (see Finney, 1947). Since the probit x is related to the proportion q so that $dq/dx=z$, the ordinate of the normal curve corresponding to the proportion q, the sampling variance of x is given by

$$V(x)=(dx/dq)^2\,V(q)=(q/lz^2+q^2/kz^2)/n.$$

The sampling variance of the difference between two probits, based on the same estimate of n, is given by

$$\begin{aligned}V(x_1-x_2)&=V(x_1)+V(x_2)-2\,\mathrm{Cov}\,x_1x_2\\&=V(q_1)/z_1^2+V(q_2)/z_2^2-2\,\mathrm{Cov}\,q_1q_2/z_1z_2\\&=\{q_1/l_1z_1^2+q_2/l_2z_2^2+(q_1/z_1-q_2/z_2)^2/k\}/n.\end{aligned}$$

If the reciprocal of this theoretical sampling variance was used to weight the probit differences derived from the data of Table 8, an χ^2 test showed that the variations between experiments were greater than expectation, but χ^2/ϕ, where ϕ is the number of degrees of freedom involved in determining the weighted mean of corresponding probit differences, showed no significant heterogeneity (Bartlett's test) due to the particular experiment involved ($P=0{\cdot}7$), the amino-acid tested for inhibition ($P=0{\cdot}9$), or its concentration ($P=0{\cdot}7$). It was thus possible to use a pooled estimate of the variance, χ^2/ϕ, based on the data as a whole, and the larger number of degrees of freedom available from this estimate allowed greater precision in the application of the t test to the significance of the weighted means.

This advantage of uniformity of variance over a wide range justified the use of the probit transformation in spite of the disadvantage that, on approaching complete inhibition of germination, the probit tends to infinity with zero weight. The significance thus becomes indeterminate unless an estimate from the regression of probit on a function of concentration of inhibitor is available. This was not necessary for the present purpose since, in practice, the significance of such large inhibitions was not in doubt. By taking the minimum expected probit X as that observed at a lower concentration of inhibitor, it was possible to calculate a minimum value for the working probit, $x=X+(q-Q)/Z$ (as defined by Finney, 1947), where Q, Z are the proportions and ordinate of the normal curve corresponding to probit X, and q is the observed proportion not germinating at the higher concentration. The weight to be used with this working probit is that corresponding to Q and not to q. It will be observed that this technique allows appropriate weight to be given even to observations where errors of random sampling lead to an estimate of q greater than 1 (e.g. with D-alanine at 20 μM, Table 8, Exp. 3). It has been applied to this and three other observations in Table 8, since their omission leads to a lower estimate for variance for the data as a whole.

RESULTS

Replacement of hydrolysed gelatin in basal medium by a synthetic mixture of amino-acids. Preliminary experiments on the toxicity of amino-acids in the gelatin-tyrosine-yeast medium, showed that leucine, isoleucine and valine, which Gladstone (1939) had shown to be inhibitory to growth under certain conditions, did not inhibit germination under the conditions used, the concentrations being those which Gladstone had found to be effective. Of the eleven amino-acids present in the simplest form of his medium, however, DL-alanine was found to be strongly inhibitory, the activity being due to the D-component. The inhibition due to excess gelatin hydrolysate was probably not due to this cause, since that due to 10^{-4} M-D-alanine was reduced from 95 to 25% by increasing the hydrolysed gelatin concentration from 0·05 to 0·30%. It is probable that the inhibition due to high concentrations of hydrolysate was largely due to glycine; at 10 mM it reduced germination in 0·25% hydrolysate from 70 to 35% compared with a reduction to 50% when the concentration of hydrolysate was doubled. Taking 24% as the glycine content of gelatin (Block & Bolling, 1945), this corresponded to an increase of 8 mM in glycine concentration.

Since it now seemed likely that failure of a large proportion of spores to germinate in a synthetic amino-acid medium was due to DL-alanine, germination was tested in a medium containing eighteen amino-acids, including threonine, at the concentrations used by Gladstone (1939), but with DL-alanine replaced by the L-form. Other constituents of the medium were modified according to Brewer *et al.* (1946). Table 1 shows that this medium allowed a slow fall in spore count. In this medium the fall was much more rapid with the addition of a preparation of yeast adenylic acid, which was used at this stage before it was realized that its activity was probably due to its content of free adenosine. In buffer the fall was much slower and was not markedly stimulated by the adenylic acid. Although the combined count of vegetative cells and spores also fell slowly, the fall in spore count has been regarded as the best measure of germination, since at 0·5 hr. when no significant fall in $S+V$ count had occurred, the spore count in the best medium had fallen by over 90%. The fall in combined count on prolongation of incubation showed that a significant proportion of the heat-sensitive forms slowly became non-viable. This applied especially to those media which did not support growth (as shown by the count at 73 hr.) so that in these media the proportion

of spores remained high. Nevertheless, it would be misleading to take the fall in the proportion of spores as an index of germination, since this proportion may increase, as in the 'eighteen amino-acids' medium, with adenylic acid, due to a fall in the $S+V$ count while the number of spores actually remains practically constant.

medium of Exp. 2, containing aspartate and glutamate in addition, but the three excluding glycine were as effective as that basal medium.

If the concentrations of either alanine or tyrosine were increased above 0·5 mM, the germination increased but about 1 % spores did not germinate, even at 1–5 mM. At submaximal concentrations the

Table 1. *Germination and growth of* Bacillus anthracis *in a synthetic medium*

(The 'eighteen amino-acids' medium was that of Gladstone (1939), including salts and glucose, with replacement of DL-alanine by L-alanine ($5{\cdot}6\times10^{-4}$ M), and the addition of DL-threonine (3×10^{-5} M), aneurin (10^{-7} M), $CaCl_2$ (5×10^{-4} M), and $NaHCO_3$ (10^{-2} M). Yeast adenylic acid (y.a.a.), where added, was 10^{-5} M.

The inoculated media, 10 ml. in 6 in. × ¾ in. tubes, were incubated for the first 5 hr. in a water bath, at 35·0±0·1°, samples of 1·0 ml. being removed at intervals for counting. Subsequently the remainder of the culture was aerated by sloping on a rocker in the hot room in such a way that the tubes passed in a vertical plane through 35 oscillations/min. of amplitude about 5°, the minimum slope being as nearly horizontal as possible.

$S+V$ = viable count of spores and vegetative cells; S = spore count.)

	M/30 phosphate pH 7·3				'Eighteen amino-acids'			
	No y.a.a.		With y.a.a.		No y.a.a.		With y.a.a.	
Time (hr.)	$(S+V)$	(S)	$(S+V)$	(S)	$(S+V)$	(S)	$(S+V)$	(S)
	Counts ($\times10^3$)							
0·0	8·6	8·7	8·4	8·4	8·3	7·5	8·0	6·5
0·5	7·5	7·6	8·0	6·2	7·3	5·9	7·7	0·5
1·5	7·2	5·9	6·7	4·1	6·1	3·1	6·0	0·1
3·0	5·9	5·4	6·1	4·6	5·9	2·5	3·8	0·1
5·0	5·8	5·0	5·7	4·2	4·4	1·0	4·1	0·1
	Counts ($\times10^3$)				Counts ($\times10^7$)			
72	—	3·4	—	2·0	—	7·5	—	4·7

Determination of essential amino-acids. It was now comparatively simple to determine the amino-acids essential for germination by omission of groups of them in turn as shown in Table 2. In Exps. 1 and 2 the basal media were adequate, and the fall in spore count was not increased by any of the thirteen amino-acids added. The inhibition by D-alanine was shown in Exp. 1, and leucine, isoleucine and valine were together partially inhibitory in the basal medium of Exp. 2. Exp. 3 shows that the group containing glycine and L-alanine was effective with phenylalanine and tyrosine as the only amino-acids in the basal medium, but aspartate and glutamate were ineffective. This latter mixture was also ineffective in the presence of glycine and L-alanine (data not tabulated). The inhibitory effect of glycine was more marked in these mixtures containing few amino-acids, so that in this experiment where it was present in both effective mixtures little germination was observed in 0·5 hr. and it was necessary to prolong the experiment for 2 hr. before more than 50 % germination was observed. Exp. 4 shows that L-alanine and tyrosine together were effective, phenylalanine being stimulatory in their presence and glycine inhibitory. These effects were shown most clearly after 1 hr. incubation. The four amino-acids together were less effective than the basal

actual germination observed was somewhat variable, but a typical experiment (Table 3) showed that 100–150 μM was adequate for over 50 % germination. In another experiment with 300 μM-alanine, 300 μM-tyrosine and 1 μM-adenosine (now used in place of adenylic acid) the germination, 75 %, was practically as great as the 90 % observed in the gelatin-tyrosine medium with the addition of the same concentration of adenosine. A tenfold dilution of the CCY medium of Gladstone & Fildes (1940) was rather more effective, only 1 % of the spores remaining heat resistant as with higher concentrations of alanine and tyrosine. With tryptic digest of beef, on the other hand, even at a dilution tenfold greater than that usual in culture media, the germination of the small inocula used was so rapid that the spore count fell about 75 % during the 1–2 min. required for mixing the inoculum with medium at 35° and transferring a sample to the diluent at 60°. Since no significant fall occurred with the other media under these conditions, it must be concluded that the broth contains nutrients, so far unidentified, which can increase the rate of germination much beyond that attainable with those nutrients now known to be effective.

Inessential components of the medium. The omission of aneurin, glucose or bivalent cations from the

Table 2. *Amino-acid requirements for germination*

(All basal media contained salts and aneurin as in Table 1.)

Exp. no.	Time (hr.)	Basal medium: Constituents	Basal medium: Concn. (μM)	Additions: Substances	Additions: Concn. (μM)	Spores not germinated: (%)	Spores not germinated: (S.D.)
1	0·5	As in Table 1 with omission of cystine, methionine and tryptophan; with yeast adenylic acid	5	None		4·9	1·0
				L-Cystine	250	4·7	1·0
				DL-Methionine	200	10·9	1·5
				Two above		6·3	1·1
				Two above + DL-Serine + DL-Tryptophan	1000 + 1200	6·4	1·1
				Four above + D-alanine	280	88·9	3·1
2	0·5	Glycine	3300	None		25·3	1·7
		L-Alanine	560	DL-Leucine + DL-Isoleucine + DL-Valine	570 + 380 + 130	63·8	3·8
		DL-Aspartate	750				
		DL-Glutamate	300	L-Arginine + L-Histidine + DL-Lysine	500 + 250 + 270	37·0	2·9
		DL-Phenylaline	300				
		DL-Tyrosine	280	DL-Threonine + DL-Proline + DL-Hydroxyproline	30 + 440 + 380	41·8	3·0
		Yeast adenylic acid	2	Nine above		46·7	3·2
3	2·0	DL-Phenylalanine	300	None		68·7	3·5
		DL-Tyrosine	280	Glycine + L-Alanine	3300 + 560	34·0	2·5
		Yeast adenylic acid	2	DL-Aspartate + DL-Glutamate	750 + 300	73·8	3·7
				Four above		32·2	2·4
4	1·0	DL-Tyrosine	280	None		90·4	4·4
		Yeast adenylic acid	2	Glycine	3300	93·5	4·5
				L-Alanine	560	39·9	2·9
				DL-Phenylalanine	300	89·8	4·4
				Glycine + L-Alanine	3300 + 560	77·9	4·0
				Glycine + DL-Phenylalanine	3300 + 300	92·1	4·5
				L-Alanine + DL-Phenylalanine	560 + 300	10·7	1·5
				Three above		55·3	3·4
	1·0	As Exp. 2		None		12·9	1·6

Table 3. *Effect of concentrations of* L-*alanine and tyrosine on germination*

(The basal medium contained glucose, salts and aneurin as in Table 1, together with $1·5 \times 10^{-6}$ M-yeast adenylic acid.)

DL-Tyrosine (μM)...	0	10	30	100	300
L-Alanine (μM)	Spore count (in 0·05 ml.) after 30 min. at 35°				
0	588	550	600	552	429
	607	—	—	—	—
50	565	600	581	148	94
150	483	478	386	91	39
500	469	472	248	66	16

medium had no significant effect in an experiment in which the total number of viable organisms, 98 ± 3 % spores ($P = 0·95$) initially, fell by 32 ± 4 % in media permitting a fall in spore count of 90 ± 2 % independently of the presence or absence of these constituents. In the absence of any one of the remaining constituents, adenosine, L-alanine or tyrosine, germination was much reduced, the $S + V$ count falling to $75·5 \pm 4$ % of the initial $S + V$ count and the spore count to 67 ± 4 %. This experiment confirms the cause in the fall in spore count as germination even in the simplest medium promoting such a fall. Replacement of the phosphate buffer by 25 mM-sodium bicarbonate in equilibrium with 5 % carbon dioxide in air, showed that phosphate was not essential but was probably stimulatory, giving 98 % germination compared with 67 % in medium

buffered by sodium bicarbonate, though the corresponding blank figures for the buffers alone were 12 and 31 % respectively.

Replacement of tyrosine by analogues. Table 4 shows a typical experiment on the effect of replacing tyrosine by phenylalanine or dihydroxyphenylalanine. Tyrosine was active at 150 μM, and the other two inactive at this level, but active at 500 μM. In all cases the D-form present in the DL-mixture appeared to have no marked effect on the activity. Table 5 shows the effects of some other analogues of tyrosine, all showing some activity. The case of adrenaline, with activity of the same order as phenylalanine, calls for special comment since it showed that an unsubstituted α-amino group was not essential for activity. Controls showed that these compounds influenced only the spore count after incubation, variations in the final $S+V$ count and in the initial $S+V$ and spore counts being within the errors of random sampling.

Table 4. *Effects of concentration and optical form of aromatic amino-acids on germination in the presence of adenosine and* L*-alanine*

(The basal medium contained 33 mM-phosphate, pH 7·3; 2 μM-adenosine and 500 μM-L-alanine. Dihydroxyphenylalanine was sterilized as 5 mM solution in 0·02M-HCl by Seitz filtration just before use.)

Concn. of added amino-acid (μM)	Spore count (in 0·05 ml.) after 30 min. at 35° with: Phenylalanine L-	Phenylalanine DL-	Tyrosine L-	Tyrosine DL-	Dihydroxyphenylalanine L-	Dihydroxyphenylalanine DL-
500	286	313	178	183	337	280
150	490	425	332	255	555	441
50	536	517	539	438	552	459
0	494; 468; 430					

Table 5. *Effects of analogues of tyrosine on germination in the presence of adenosine and* L*-alanine*

(The basal medium contained 33 mM-phosphate, pH 7·3; 6 μM-adenosine and 500 μM-L-alanine. Just before use, the materials added were dissolved in 0·02N-HCl, Seitz filtered and added to the basal medium containing the amount of NaOH required for neutralization.)

Addition to basal medium (500 μM)	Viable counts (in 0·05 ml.) 0 min. $S+V$	0 min. Spore	30 min. $S+V$	30 min. Spore
None	596	546	494	441
Diiodotyrosine	596	501	460	341
Tyramine	579	525	480	295
Thyroxine	582	537	485	271
DL-Dihydroxyphenylalanine	579	531	497	231
Adrenaline	593	504	502	192
DL-Phenylalanine	603	527	490	151
DL-Tyrosine	586	512	486	96

Specificity of alanine. The stereochemical specificity of the enantiomorphs of alanine has already been indicated since it was necessary to replace DL-alanine by its L-component before germination was observed in a synthetic medium. Table 6 shows that L-alanine was not replaceable by β-alanine, nor by pyruvate or lactate, with or without ammonium chloride as a source of nitrogen. None of the last three was inhibitory in the presence of L-alanine, so that inhibition, as by D-alanine, did not occur with the corresponding form in DL-lactate. Reversal of D-alanine inhibition by L-alanine is shown in Table 7, a ratio of 10–30 molecules of promoter to one molecule of inhibitor giving about 50 % germination.

Table 6. *Specificity of* L*-alanine in promoting germination in the presence of adenosine and tyrosine*

(The basal medium contained 33 mM-phosphate, pH 7·3; 1·5 μM-adenosine and 500 μM-tyrosine. The concentrations of the additions to the basal medium were NH_4Cl and pyruvate, 5 mM; DL-lactate and β-alanine, 2·5 mM; L-alanine, 0·5 mM.)

Additions to basal medium	Spore counts (in 0·05 ml.) 0 min.	30 min.
None	664	547
NH_4Cl	647	589
Lactate	653	596
Lactate + NH_4Cl	637	572
Pyruvate	668	589
Pyruvate + NH_4Cl	633	577
β-Alanine	661	480
L-Alanine	626	62
L-Alanine + NH_4Cl	641	34
L-Alanine + lactate	659	35
L-Alanine + pyruvate	645	166

Table 7. *Inhibition of germination by* D*-alanine*

(The basal medium contained 33 mM-phosphate, pH 7·3; 5 μM-adenosine and 500 μM-DL-tyrosine.)

Alanine (μM) L-	Alanine (μM) D-	Other additions	Spore count (in 0·05 ml.) 0 min.	30 min.
0	0	None	974	818
0	15	None	—	898
0	50	None	—	908
0	150	None	914	916
500	0	None	—	136
500	0	Brucine (20 μg./ml.)	845	248
500	0	Strychnine (20 μg./ml.)	897	231
500	15	None	—	403
500	50	None	—	655
500	150	None	—	836
1500	0	None	—	89
1500	15	None	—	142
1500	50	None	—	227
1500	150	None	—	461
5000	0	None	926	84
5000	15	None	—	73
5000	50	None	—	111
5000	150	None	910	385

Controls showed that traces of the alkaloids used in resolution could not be responsible for the inhibition, since in the presence of 500 μM-L-alanine, 15 μM-D-alanine (1·35 μg./ml.) reduced germination by more than twice as much as 20 μg./ml. brucine or strychnine.

Inhibition by other amino-acids. Indications of inhibition by glycine and by leucine, isoleucine and valine together had already been seen in determining the constituents responsible for germination in the original synthetic medium containing nineteen amino-acids. Observations were therefore made (Table 8) with a number of DL- and (where available) D-amino-acids, in a search for further examples of inhibition correlated with stereochemical form. Highly significant inhibitions ($P < 0·02$ for equal or greater effect in the same direction due to chance) were observed both at 1·5 and 5 mM only with glycine and DL-methionine. DL-Cysteine and D-valine were significantly inhibitory at 5 mM, but not at 1·5 mM, while with D-glutamate and DL-proline the inhibition at 5 mM was barely significant ($P \approx 0·05$). At this concentration D-leucine, DL-lysine, D-phenylalanine and DL-threonine showed no significant effect. Since D-alanine was significantly inhibitory ($P = 0·01$) even at 5 μM, the problem of the weaker inhibitions and their relation to stereochemical form has not been pursued further.

Table 8. *Inhibition of germination by amino-acids*

(The basal medium contained 33 mM-phosphate, pH 7·3; 2 μM-adenosine; 300 μM-L-alanine and 300 μM-DL-tyrosine.)

Exp. no.		1	2	3	4	5	6	7
Initial spores (in 0·05 ml.)		1	12	3	17	10	12	10
No. replicates	Mean count	1017	650	816	516	570	493	402

Added to medium		Spore count after 30 min. at 35°							Probit final spores		Probability of greater difference in one direction due to chance	Spores corresponding to probits (%)	
Amino-acid	Concn. (μM)								Weighted mean	S.E.		Mean	Difference
None	—	216	250	307	206	75	174	45	4·40	0·14	—	27	± 7
		204	237	287	221	95	154	33					
		198	—	—	208	—	—	—					
									Weighted mean difference				
D-Alanine	1·5	—	542	—	330	—	156	—	+0·39	0·32	0·12	42	+14
	5	—	576	728	406	—	252	—	+0·85	0·35	0·010	60	+33
	20	—	—	869	416	—	—	—	>1·57	0·38	<0·001	83	+56
	60	944	—	—	—	—	—	—	+2·29	1·33	0·026	96	+69
	200	904	—	—	—	—	—	—	+2·05	0·98	0·024	95	+68
Glycine	1500	630	—	—	329	—	—	—	+0·94	0·32	0·003	63	+36
	5000	1017	—	—	427	—	—	—	>1·95	0·36	<0·001	91	+64
DL-Methionine	1500	—	—	574	—	186	—	—	+0·71	0·32	0·017	54	+27
	5000	—	—	783	—	352	—	—	>1·49	0·36	<0·001	81	+54
D-Valine	1500	283	257	—	—	—	—	—	+0·18	0·24	0·23	33	+ 6
	5000	463	646	—	—	—	—	—	>1·07	0·25	<0·001	68	+41
DL-Cysteine	1500	—	—	165	—	96	—	—	−0·31	0·24	0·11	18	− 9
	5000	—	—	606	—	480	—	—	+1·28	0·46	0·004	75	+48
D-Glutamate	1500	93	307	—	—	—	174	55	−0·08	0·21	0·35	24	− 3
	5000	217	341	—	—	—	154	36	+0·38	0·22	0·047	41	+14
DL-Proline	1500	—	—	386	159	—	254	49	+0·16	0·22	0·24	33	+ 6
	5000	—	—	695	176	—	412	113	+0·46	0·29	0·062	44	+17
D-Leucine	5000	195	194	—	—	—	—	—	−0·10	0·24	0·34	24	− 3
DL-Lysine	5000	—	—	216	122	—	343	44	−0·13	0·22	0·28	23	− 4
D-Phenylalanine	5000	272	165	—	—	—	—	—	−0·01	0·23	0·49	27	0
DL-Threonine	5000	—	—	—	98	262	314	—	+0·17	0·25	0·26	33	+ 6

DISCUSSION

The most striking feature of this work is the high specificity of L-alanine in promoting germination and the inhibition by its enantiomorph. No such specificity was shown by tyrosine which was replaceable in some degree by related compounds, the carboxyl and unsubstituted amino groups not being essential for activity. The activity of the L-form was

not changed significantly when supplied by the synthetic DL-compound.

The small molecular ratio of D-alanine required to inhibit the action of the L-form was especially remarkable since inhibition of microbiological processes by structural analogues usually requires many molecules of inhibitor to one of promoter, e.g. 5000 for sulphonamides and *p*-aminobenzoate (Woods, 1940). In the metabolism of amino-acids by animal tissues, there are well-known examples of the occurrence of inhibitions by enantiomorphs at concentrations comparable to that of the substrate, e.g. synthesis of glutamine (Krebs, 1935); deamination of histidine (Edlbacher, Baur & Becker, 1940). In bacterial chemistry, on the other hand, the use of synthetic DL-amino-acids as standards in microbiological assay (for review see Snell, 1945*a*), to avoid active impurities of biological origin, has shown that, in general, only the L-forms have been metabolized, the antipodes being inert rather than inhibitory. In some cases the D-form may act as a supplement for the L-form, but this usually occurs only to a limited extent and requires the presence of sufficient L-form to initiate growth (for review see Rydon, 1948). The only species of micro-organisms which have so far been shown to be inhibited by D-amino-acids are *Lactobacillus arabinosus* (*Lb. plantarum*) (Fling & Fox, 1945) and *Escherichia coli* (Kobayashi, Fling & Fox, 1948). Acid production by the former, growing in a synthetic medium, was inhibited by D-leucine and D-valine at concentrations of the order 200-fold that of the L-forms essential for growth. The latter, growing in nutrient broth, was inhibited 80–90 % by 50 mM-D-leucine, 80 mM-D-valine, 120 mM-glycine or 140 mM-D-alanine. *Lb. arabinosus* was also inhibited by glycine at such high concentrations. The present work on germination shows that these amino-acids, except D-leucine, were inhibitory at 5 mM, as were DL-cysteine and DL-methionine also, but D-alanine was outstanding by inhibiting even at 5 μM. The inhibition of growth of *B. anthracis* by unbalanced concentrations of leucine, isoleucine and valine was not due to the D-forms (Gladstone, 1939), but with similar inhibitions due to norleucine, serine or threonine the effect of antipodes was not investigated, nor was this done with the inhibition of growth of *Streptococcus lactis* investigated by Snell & Guirard (1943), who also found glycine and β-alanine to be inhibitory. With this last-named organism, and with *Lb. casei* (Snell, 1945*b*), D-alanine not only failed to inhibit, but at a sufficient concentration, eliminated the need for pyridoxine in growth and in reversing the inhibitions by other amino-acids above mentioned.

Even in the case of *B. anthracis*, the inhibition by D-alanine was confined to the germination of spores, and growth was not inhibited significantly. Gladstone (1939) grew six strains satisfactorily in an amino-acid medium containing DL-alanine, and although the strain used in the present work required supplementation of Gladstone's medium with aneurin, growth was then obtained in the presence of DL-alanine, even though the proportion of spores which germinated was not detected by viable counts.

It may be concluded that inhibition by stereoisomers of amino-acids is rare in bacteriological chemistry and that elucidation of the reason for this strong inhibition by D-alanine, the simplest amino-acid capable of showing optical isomerism, might have some bearing on the design of antibacterial agents of therapeutic value (cf. Work, 1948). In this connexion, it may be recalled that only the D-form of penicillinamine hydrochloride gave rise to active penicillins on condensation with appropriate oxazolones (quoted by Du Vigneaud, Carpenter, Holley, Livermore & Rachele, 1946). At present no reason can be offered for the importance of the two forms of alanine in promoting and inhibiting germination since existing knowledge of the metabolism of spores (Ruehle, 1923; Cook, 1931; Tarr, 1933; Virtanen & Pulkki, 1933; Keilin & Hartree, 1947) does not include their action on amino-acids.

SUMMARY

1. The germination of spores of a virulent strain of *Bacillus anthracis* has been studied by means of viable counts, and the amino-acid requirement has been determined.

2. Slow germination occurred in phosphate buffer at pH 7·3, but was greatly stimulated by 500 μM-L-alanine, 500 μM-L-tyrosine and 2 μM-adenosine together.

3. L-Alanine was not replaceable by related compounds and its action was strongly inhibited by D-alanine at a concentration 0·03 times that of its antipode. The inhibition was reversed by increasing the proportion of the L-form.

4. L-Tyrosine was replaceable by related compounds with little decrease in efficiency, none of the groups—phenolic hydroxyl, amino and carboxyl—being essential for activity. Its activity was not suppressed when supplied as DL-tyrosine.

5. Of seventeen other amino-acids tested, none showed significant stimulation of germination. Glycine, DL-methionine, DL-cysteine and DL-valine showed inhibition, but only at concentrations 1000 times that at which D-alanine inhibited.

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Studies in Vitamin A

14. THE ALLEGED MOBILIZATION OF VITAMIN A BY ADRENALINE

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In 1940 Young & Wald reported that the injection of adrenaline into a rabbit was followed by a very marked increase in the blood vitamin A level, which reached a maximum approximately 30 min. after injection. In view of its possible importance in helping to elucidate the mode of action of vitamin A and the possible clinical implications, the effect has been reinvestigated using both rats and rabbits as experimental animals.

EXPERIMENTAL

Animals. Rabbits were kept in a constant environment and given an unvarying diet of Lever's cubes and outer leaves of cabbage; rats were maintained similarly except that they were not given cabbage.

Dosing. The required dose of adrenaline was injected intraperitoneally into rats and intravenously into a marginal ear vein of rabbits.

Collection and examination of blood. The samples of rat blood were withdrawn by cardiac puncture using a method previously described (Glover, Goodwin & Morton, 1946). The samples of rabbit blood (approx. 10 ml.) were withdrawn without anaesthesia from a marginal vein of the ear not used for the injection of adrenaline.

In most experiments the plasma was separated from the red cells and examined for vitamin A using the method developed in this laboratory (Glover *et al.* 1946; Goodwin & Gregory, 1948). In some experiments whole blood was used and extracted according to the method of Young & Wald (1940), but the extract was examined for vitamin A using our method.

RESULTS

Rabbits. The results of the rabbit experiments are collected in Table 1. It will be seen that adrenaline has no appreciable effect on the vitamin A plasma levels of rabbit, and an analysis of the data using the t test indicated that the differences are not significant.

Young & Wald (1940) used whole blood for their vitamin A tests, but as it is now well established that under normal circumstances no vitamin A exists in the red cells, most of our experiments were carried out on blood plasma. There was, however, a possibility that adrenaline mobilized vitamin A into the red cells and that this would account for Young & Wald's results. To check this, two experiments were carried out in which whole blood instead of plasma was examined for vitamin A; again no mobilization was apparent.

Rats. When rats were used the experimental approach had to be altered slightly. The volume of

Table 1. *The action of adrenaline on the blood levels of vitamin A in rabbits*

Animal no.	Dose of 1/1000 adrenaline (ml.)	Interval between injection and withdrawal of blood (min.)	Vitamin A plasma values (i.u./100 ml.) Before injection	After injection
C	1·0	120	215	226
120	1·0	30	213	211
120	0·5	30	213	193
134	1·0	10	224	221
81	1·0	35	221	252
113	1·0	10	218	234
161	0·5	30	204	180
167	0·5	30	82	71*
130	0·5	30	55	46*

* The values in these two experiments are those obtained using whole blood instead of plasma only.

blood required for each test was more than it was possible to draw from the same animal before and after injection of adrenaline. Consequently, groups of rats (about six per group) of the same sex, age and nutritional background were used; one group was injected with adrenaline and the other served as a control. The vitamin A plasma level of each rat was determined separately and the mean values for the two groups compared. It will be seen from Table 2 that no significant differences could be demonstrated between the control groups and those treated with adrenaline. It should be noted that the variations between the values obtained on rats in a single group are quite small, and could thus in no way mask an effect as large as that encountered by Young & Wald (1940); to illustrate this the individual results are quoted for Exp. 4 (Table 2).

Table 2. *The action of adrenaline on the plasma levels of vitamin A in rats*

Exp. no.	Dose of 1/1000 adrenaline (ml./rat)	No. of rats Control group	Test group	Interval between injection and withdrawal of blood (min.)	Mean vitamin A plasma levels (i.u./100 ml.) Control group	Test group
1	0·5	7	7	30	49·9	49·8
2	0·5	10	3	60	35·8	40·4
3	0·5	8	4	15	38·0	45·7
4	0·5	4	4	14	61·7	61·7*
					64·3	66·8
					66·3	68·8
					57·1	61·7
				Mean	**62·3**	**64·7**

* The results of Exp. 4 are recorded in detail to illustrate the usual variations found between plasma levels of rats of the same group.

DISCUSSION

Owing to the existence of a blood-liver equilibrium the plasma levels of vitamin A alcohol (the functional form) are only slightly raised even after large doses of vitamin A (Glover *et al.* 1946); thus any substance which can mobilize vitamin A alcohol into the blood from the mainly esterified liver reserves might be of considerable importance clinically. Clausen, Baum, McCoord, Rydeen & Breese (1940) claimed that ethanol mobilized vitamin A in dogs and later the same group of workers (Clausen, Breese, Baum, McCoord & Rydeen, 1941) stated that drinking liquor to the extent of 'the usual "social" evening common in this country' (U.S.A.) raised the vitamin A plasma levels of humans. Brenner & Roberts (1943) also found a rise in humans. However, not only have British investigators failed to observe any rise after administration of ethanol (Yudkin, 1941; Hume & Krebs, 1949), but the increase recorded by the American investigators was almost entirely due to esterified vitamin A (Clausen, Baum, McCoord, Rydeen & Breese, 1942), so that any such rise would apparently serve no useful therapeutic purpose. This raised the question whether the surprisingly large increase in the vitamin A level of plasma after adrenaline injections reported by Young & Wald (1940)—a doubling within 30 min.—was in the alcohol or the ester fraction.

We could not test this point, however, as we were unable to reproduce Young & Wald's results; no mobilization of vitamin A was apparent in either plasma or red cells. It is impossible at the moment to offer any explanation of these contradictory results.

SUMMARY

1. The claim that intravenous injection of adrenaline into rabbits mobilizes liver vitamin A into the blood has not been confirmed.

2. Similar negative results were obtained with rats.

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